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14. ABSTRACT

The objective of this proposal is to establish the graft preservation and immunomodulatory effects of our MP-BMPS/HBOC system in a pre-clinical large animal CTA model. We will specifically address the following specific aims in a porcine vascularized musculo-adipo-cutaneous flap model:

Aim 1: Does MP-BMPS/HBOC allow prolongation of CIT without significant cellular damage to the allograft?
Aim 2: Does the MP-BMPS/HBOC minimize the effects and incidence of ischemia-reperfusion injury at revascularization?
Aim 3: What is the effect of MP-BMPS/HBOC on the immune profile of various flap tissues after transplantation?

15. SUBJECT TERMS
Machine perfusion, limb transplantation, graft preservation, MP-BMPS/HBOC, ischemia, cold ischemia

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1. INTRODUCTION:

This study was an expansion our previous experience [1-7] with machine perfusion (MP) in combination with a newly developed hemoglobin based oxygen carrier (HBOC) solution under subnormothermic (21°C) conditions as a way to enhance organ and tissue preservation by providing effective ex-vivo oxygenation.

The experiments were performed on time and within budget. These experiments were developed as a proof-of-concept stage for the utilization of this new preservation technology in composite tissue allotransplants (CTAs).

The experiments were successful in showing the role and the effectiveness of ex-vivo oxygenation in CTAs and the superiority of this approach when compared to the cold static preservation, which is the current standard of care.

2. Key words

Machine perfusion (MP), cold static preservation (CSP), cold ischemia time (CIT), hemoglobin based oxygen carrier (HBOC), composite tissue allotransplants (CTAs), subnormothermic (SN), vascularized composite allotransplantation (VCA), vertical rectus abdominis (VRAM), University of Wisconsin solution (UW), superior epigastric artery (SEA), cytokines, metabolomics, ischemia-reperfusion injury (IRI), Belzer Machine Perfusion Solution (BMPS).

3 Summary/Specific Aims and Accomplishments

3.1. SPECIFIC AIM 1: Determine if the MP-BMPS/HBOC allows prolongation of CIT without significant cellular damage to the allograft.

This 1st specific aim was successfully achieved. We're able to show that our VRAM graft was able to be preserved for 14 hours at 21°C with no signs of any cellular damage to the allograft. The endothelial cells within the vasculature showed no signs of any damage after an extended period of time of ex-vivo perfusion. These results are comparable with previous publications from similar approaches [8-10].

The VRAM grafts showed stable weight after this extensive time of MP. The VRAM grafts show full anatomic integrity of all tissue compartments (e.g. vascular, interstitial, skin, adipose and muscular tissue). There was no damage to the vascular endothelial cells in both arterial and venous structures.

3.2. SPECIFIC AIM 2: Determine if the MP-BMPS/HBOC system minimizes the effects and incidence of I/R injury at revascularization.

The 2nd specific aim was successfully achieved. The MP/HBOC system provided effective oxygenation during the VRAM preservation. The tissues were intact and metabolically active. The magnitude of the IRI was significantly higher in the control group (CSP), where

early apoptosis was subsequently followed by necrosis in both the adipose and muscular tissues. The control group showed early contraction bands within the muscular tissue within the initial period (4 hours). These contraction bands expanded over time and led to further apoptosis and necrosis after VRAM graft reperfusion [11, 12]. The VRAM grafts perfused by the MP/HBOS system didn't show signs of contracting bands over the extended period of preservation (14 hours) and clear features of a self-sustained and mild IRI after reperfusion.

Our transplant pathologists were able to provide a more objective assessment of the IRIs by scoring them blindly within 5 different segments of the VRAM grafts (e.g. skin, subcutaneous adipose tissue, muscle, microvasculature and large hilar vessels). This modified scoring system showed a higher score of inflammation and tissue damage in the control group (CSP).

Further histological analysis is underway. Our preliminary analysis shows a lower degree of inflammation across the board in the MP, which reinforces our previous similar findings in liver allografts.

3.3. SPECIFIC AIM 3: Determine the effect of MP-BMPS/HBOC on the immune profile of various flap tissues after transplantation.

Tissues exposed to prolonged oxygen deprivation, or "ischemia", followed by restoration of blood-flow, can display a wide range of features compatible with IRI. The lack of oxygen supply to the control group would trigger the following cycle that ultimately lead to cell death. Figure 1 [13] displays the metabolic pathways involved in persistent anoxia.

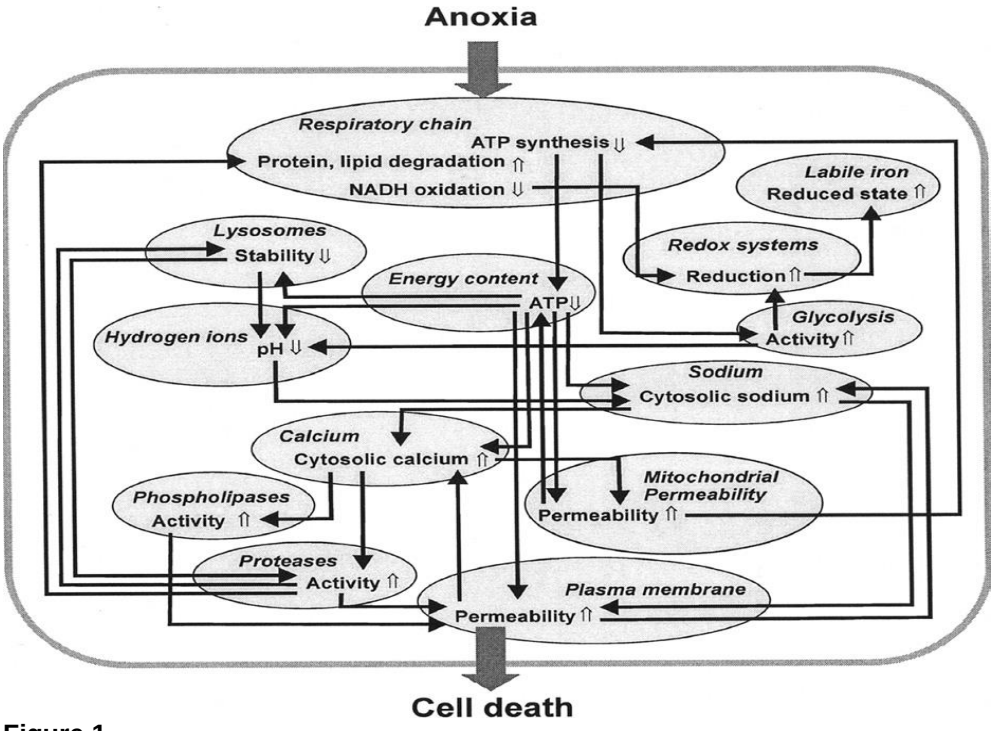


Figure 1

Tissue injury from IRI can involve both the parenchymal and the endothelial cells of the grafts being preserved ex-vivo. Figure 2 displays the pathogenic network of the inflammatory responses, where self-amplifying loops are responsible for the expansion and propagation of the immune response towards necrosis and irreversible tissue damage [13].

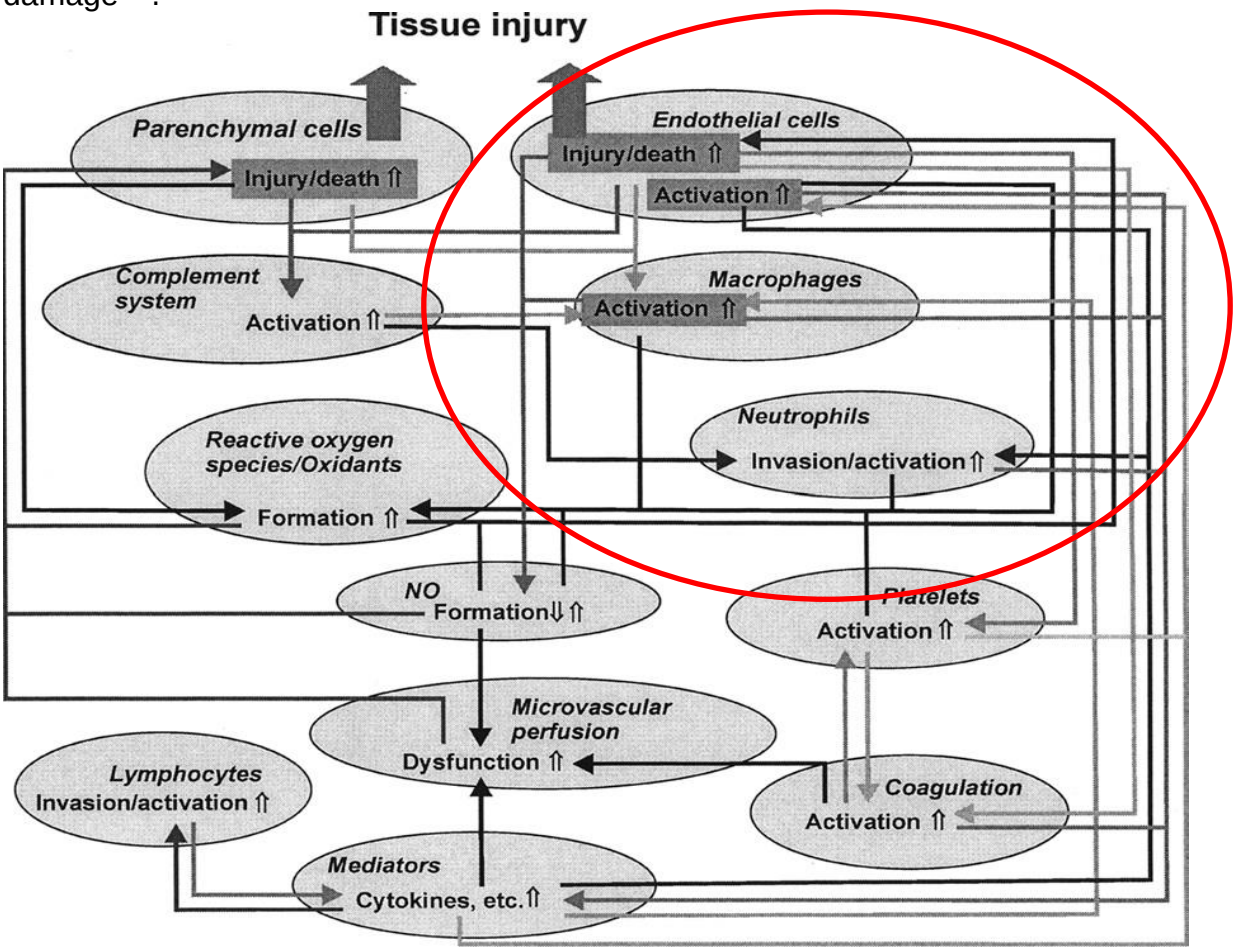


Figure 2

Our experiments revealed a significant amount of neutrophil activation after reperfusion in the CSP, where a more pronounced inflammatory response was observed when compared to the MP group. In the CSP, the restoration of blood flow after graft implantation appeared to trigger the activation of endothelial cells, which was followed by enhanced adhesion of neutrophils and platelets to the vascular endothelium. This led into increased microvascular permeability and perivascular leakage. Furthermore, a significant amount of macrophages were seen in the CSP group. The macrophages were further differentiated into histiocytes and played a significant role in the formation of calcified granulomas (calcinosis) within the muscular tissue.

The overall magnitude of the recipient's inflammatory response was significantly lower in the MP group, where reliable levels of oxygenation were sustained over the preservation period (14 hours) prior to transplantation.

Based on our initial histological analysis, the use of our MP/HBOC system has a significant beneficial effect in minimizing the magnitude of the IRIs seen immediately after graft preservation. The downstream effect on the MP/HBOC system in the immune profile of the VRAM graft is also beneficial, since the initial decrease in inflammation from the preservation stage appears to minimize further cell infiltration and the irreversible tissue damage clearly observed in the control group (CSP), which were treated according to the current standard of care for clinical preservation and transplantation.

4. Accomplishments

The surgical procedures utilized in this project were successful and no technical complications were documented with the swine vertical rectus abdominis (VRAM) myocutaneous flap. All the animals received triple immunosuppression (Tacrolimus, Mycophenolate Mofetyl and Prednisone) and there no adverse events documented during the post-operative period (7 days). All the animals were managed within USDA guidelines within an approved IACUC protocol and there were no issues related to the animal experiments.

There were several sets of data obtained from the experiments. Table 1 shows a summary of all the groups and samples collected during both stages.

Group	Intervention	Description
1. Ex Vivo Control (n=4) CSP Standard of Care	4 VRAM grafts preserved for 14 hours with UW at 4°C	Control group – baseline studies
2. Ex Vivo Study Group (n=4) Machine Perfusion	4 VRAM grafts preserved for 14 hours with MP at 21°C	Study group – developments for machine perfusion device
3. In Vivo Control (n=4) CSP Standard of Care	4 VRAM grafts preserved for 14 hours with UW at 4°C and transplanted heterotopically (cervical)	Control group – baseline for ischemia reperfusion injuries and VRAM graft viability after 7 days
4. In Vivo Study Group (n=4) Machine Perfusion	4 VRAM grafts preserved for 14 hours with MP at 21°C and transplanted heterotopically (cervical)	Study group – impact of MP in ischemia reperfusion injuries and VRAM graft viability after 7 days

Table 1

The VRAM flap recovery techniques were uneventful. The VRAM graft has medium-caliber vessels (artery and vein) feeding a sizable portion of tissue containing skin, subcutaneous adipose tissue and muscle. All VRAM grafts were flushed with cold (4°C) UW solution after cross clamp.

Figure 3. Schematic representation of the anatomical features of VRAM grafts in humans. Our experiments were based on the recovery and transplantation of the VRAM grafts though perfusion by the superior epigastric artery (SEA) vascular pedicle.

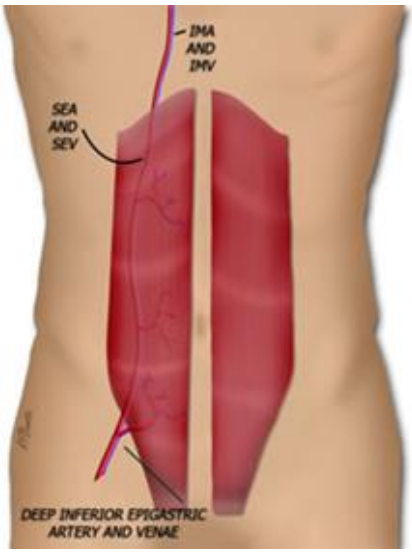


Figure 3

Figure 4. The VRAM graft outlined in the donor before graft procurement.

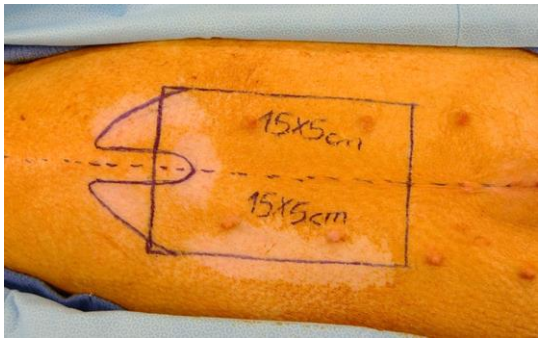


Figure 4

Figure 5. Completion of the surgical procedure to recover the VRAM graft in our swine model. The ruler is aligned with the completely isolated pedicle of the SEA.

Figure 5

These preservation and transplantation experiments with the VRAM graft were conducted in 2 separate stages. Step 1 was named ex-vivo and primarily focused on preservation and in the technical features (e.g. pressures, flow, oxygenation, temperature) involved in MP.
Step 1 – Ex-vivo stage:

Ex-vivo experiments with the VRAM grafts were conducted over a 14 hour preservation period. VRAM grafts were surgically recovered and preserved by our MP/HBOC system (n=4) while being compared to cold static preservation (CSP) (n=4) as the current standard of care.

The ex-vivo experiments were devoted to streamline all the technical aspects related to VRAM graft perfusion, since this was a new application of our technology previously and successfully performed in both porcine and human liver allografts.

Machine Perfusion of the skin flaps was performed using our initial prototype Organ Assist Liver Assist® device in combination with our HBOC solution, which contains a hemoglobin-based oxygen carrier and organ preservation solution mixed 1:3 ratio, respectively. The starting hemoglobin as measured by an ABL800flex (Radiometer, Copenhagen) blood gas analyzer was 3.4 g/dL. The baseline settings for the MP system were: 60 mmHg pressure, 21°C, FiO2 60%, sweep gas 0.3 L/min. Perfusion was initiated with an inlet pressure of 60mmHg at 1Hz pulse pressure, achieving a flow of ~10mL/min. Initial blood gas values were ~93% saturated HBOC solution at a pO_2 of ~400 mmHg. As the initial vasoconstriction from VRAM recovery subsided, the flows progressively increased over time. The MP device alters centrifugal pump speed to maintain a set pressure. After 2 hours, with flows exceeding 25ml/min, the pressure set point was lowered to 50 mmHg where it was maintained throughout the remainder of the perfusion. After 14 hours, the VRAM graft was removed from the MP device, weighted and processed for additional studies.

Step 2 - In-vivo stage – VRAM graft transplantation and the assessment of the IRIs after preservation by 2 different methods (CSP vs. MP)

The in-vivo studies were also composed by 2 groups of 4 animals each. The study group (MP) had 4 animals transplanted heterotopically (cervical implantation using the carotid

artery for inflow and the external jugular vein for venous drainage) with the VRAM allografts after a period of 14 hours of preservation where full oxygenation was provided within a low pressure and low flow protocol. The control group (CSP) had 4 animals transplanted with VRAM allografts after 14 hours of preservation with CSP as the current standard of care. Both groups were followed for 7 days. The VRAM grafts were biopsied on days 2, 4 and 7. An end-study necropsy was performed on day 7. All the animals received full immunosuppressive therapy composed by Tacrolimus, Mycophenolate Mofetil and Prednisone. All the animals had daily clinical and laboratorial assessment. Additional studies (e.g. transcriptomics, proteomics and metabolomics) were performed to assess graft viability and the impact of ischemia reperfusion injuries (IRI) suffered after this prolonged period of preservation.

Final Results from the MP experiments

Perfusion flow rates

The VRAM grafts were properly perfused with low flows and showed no signs of weight gain or endothelial cell damage during the extended time (14 hours) of MP. Figure 1 displays the VRAM graft on the PVC mesh during MP.

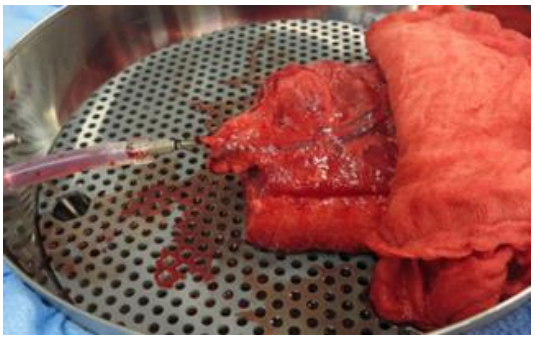


Figure 6

Figure 7 displays the flow rates (ml/min).

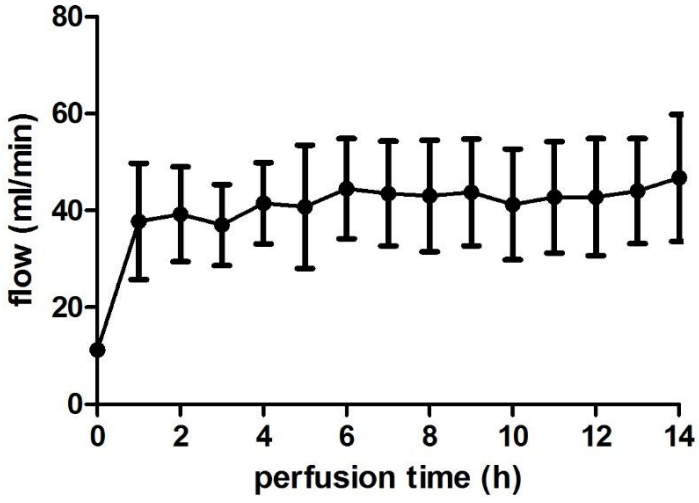


Figure 7

Perfusion pressures

The MP device was able to provide reliable pressures over the 14 hour perfusion protocol. The system was developed to provide lower than physiological arterial pressures as a way to sustain endothelial cell integrity within the macro and microvasculature. Figure 8 displays the pressures (mm/Hg) over the 14 hour period.

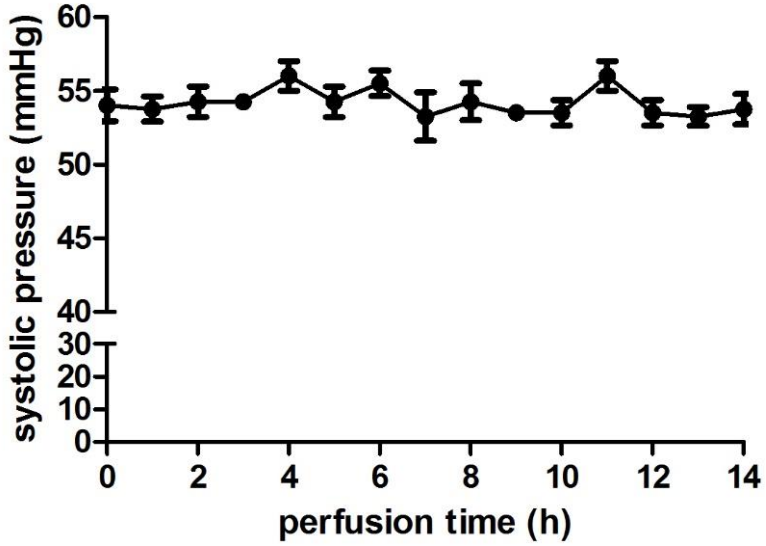


Figure 8

Oxygen delivery

The MP system in combination with our previously developed HBOC was capable to deliver adequate amounts of oxygen to the VRAM grafts over a 14 hour period at 21°C. Figure 9 displays oxygen (O_2) delivery (mlO_2/min/g tissue) over a 14 hour period.

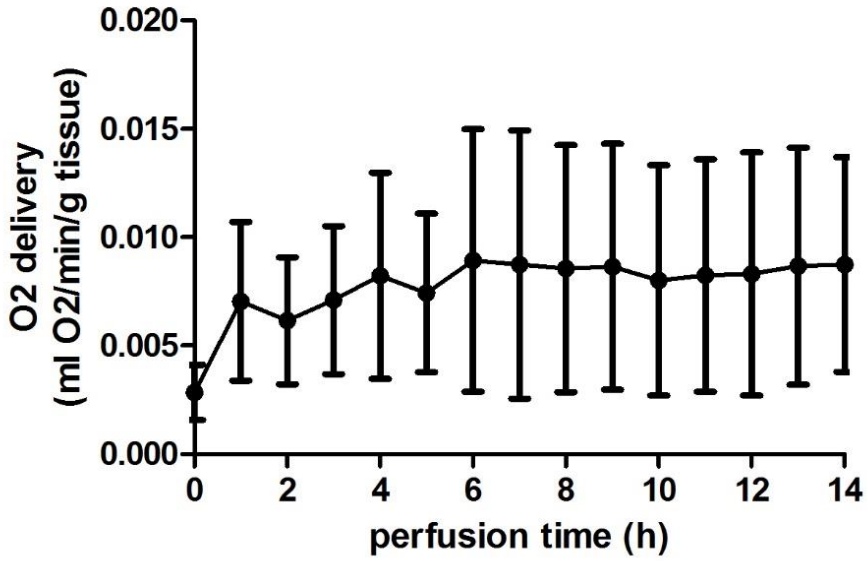


Figure 9

Indirect measurements of O_2 delivery were also assessed by measuring lactate concentration within the perfusate. Figure 10 displays lactate levels (mmol/L) in the perfusate over a 14 hour period at 21°C.

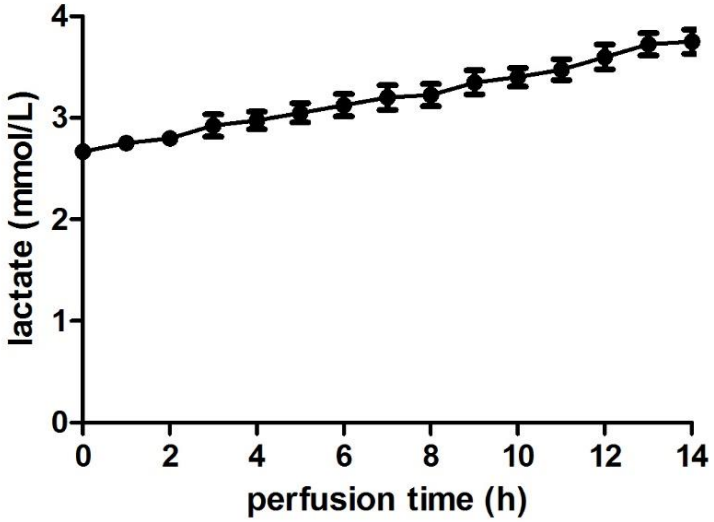


Figure 10

Figure 11 displays the pH continuously monitored in the perfusate during the MP protocol.

There were no signs of acidosis and no NaHCO3 infusions were required to sustain a physiologic (7.35-7.45) pH over a 14 hour period.

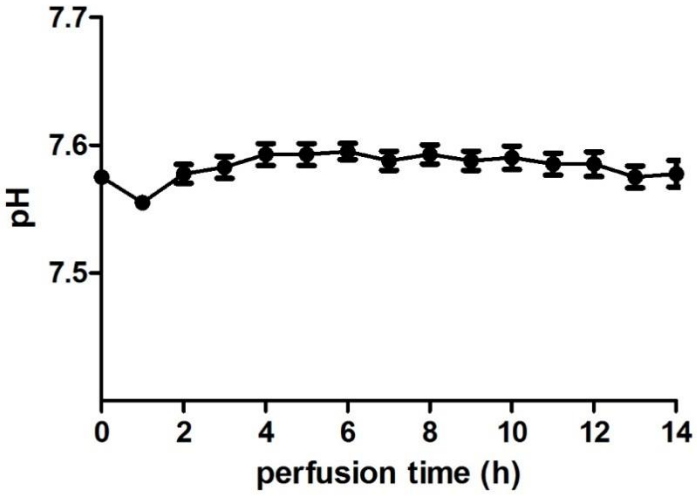


Figure 11

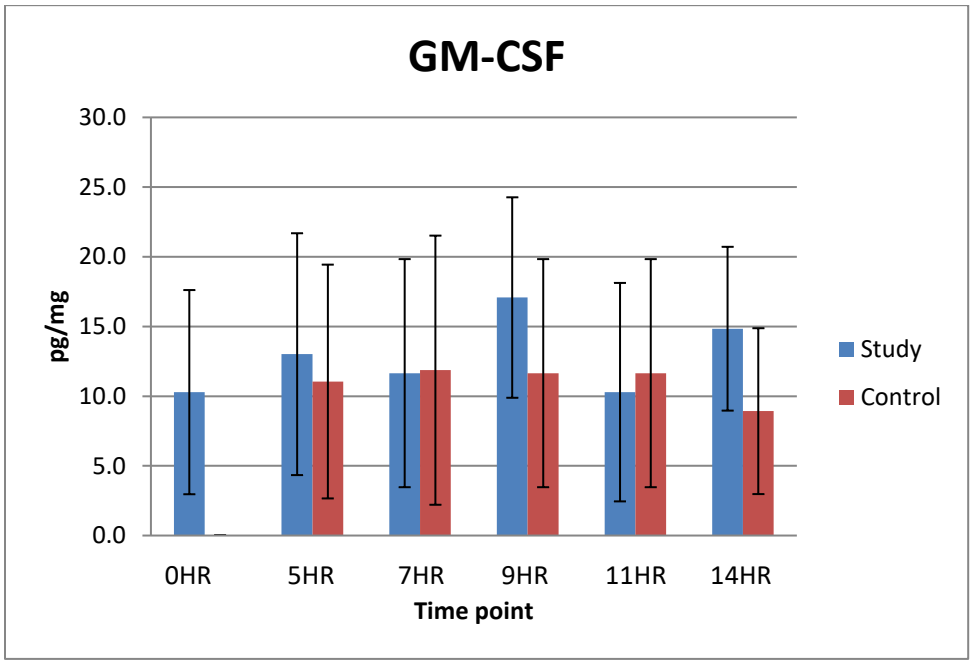
Inflammatory markers

In order to quantify the inflammatory process triggered by IRI and the imminent alloreaction against the VRAM after graft implantation, a full cytokine profile was obtained during VRAM graft preservation in both groups (MP and CSP). Subsequent samples were obtained from tissue biopsies during the post-operative period.

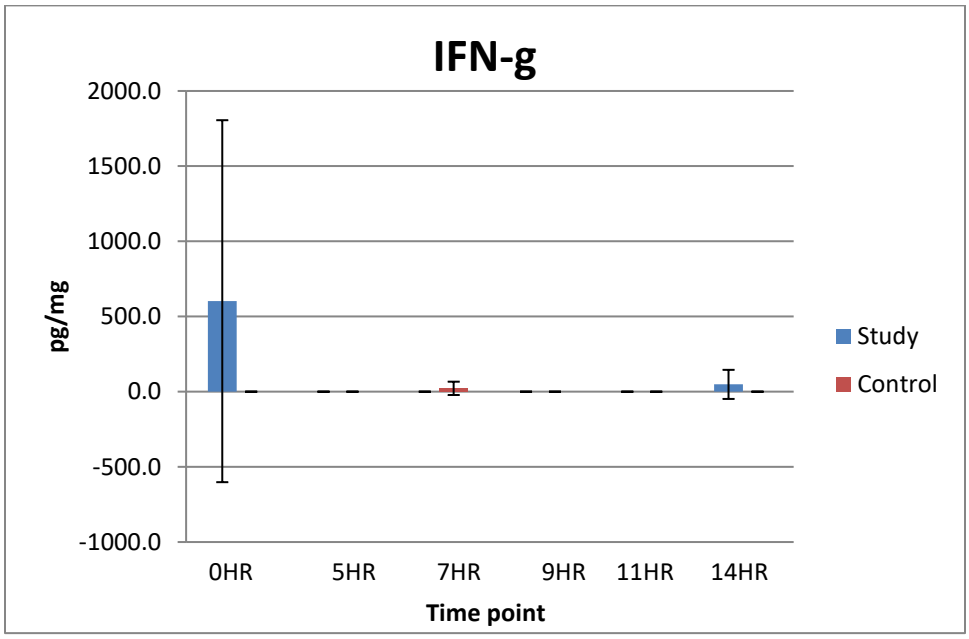
Tissue and perfusate assays of interferon IFN-γ, IL-10, IL-12/IL-23 p40, IL-1β, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α were carried out using a Luminex™ beadset from Affymetrix (Santa Clara, CA). GM-CSF, IL- 1α, IL-1RA, IL-2 and IL-18 were measured using a Luminex™ beadset from Millipore (Merck KGaA, Darmsdadt, Germany)[28]. Perfusate samples were also assessed for arterial blood gases (MP group only) and biochemical parameters. Tissue samples were normalized by protein content to account for experimental variability in cell number and protein concentration among individual samples.

The results during the ex-vivo stage (graft preservation) were the following:

1. GMCSF



2. IFN-g


IFN-g
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1000.0
500.0
0.0
-500.0
-1000.0
pg/mg
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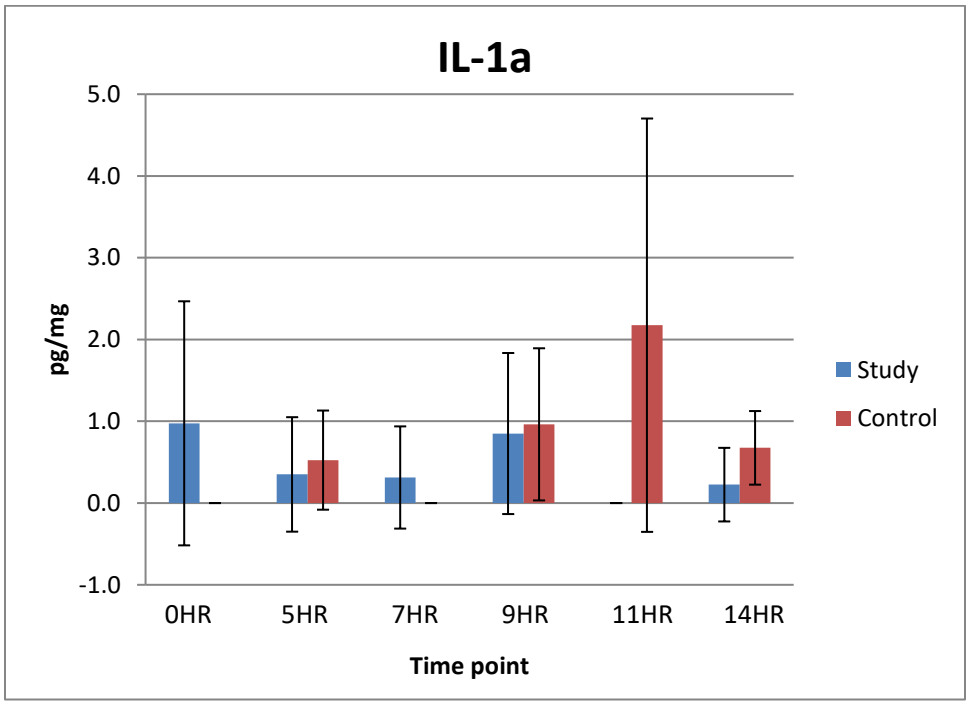
5HR
7HR
9HR
11HR
14HR
Time point
Study
Control

3. IL-1a


IL-1a
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4.0
3.0
2.0
1.0
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pg/mg
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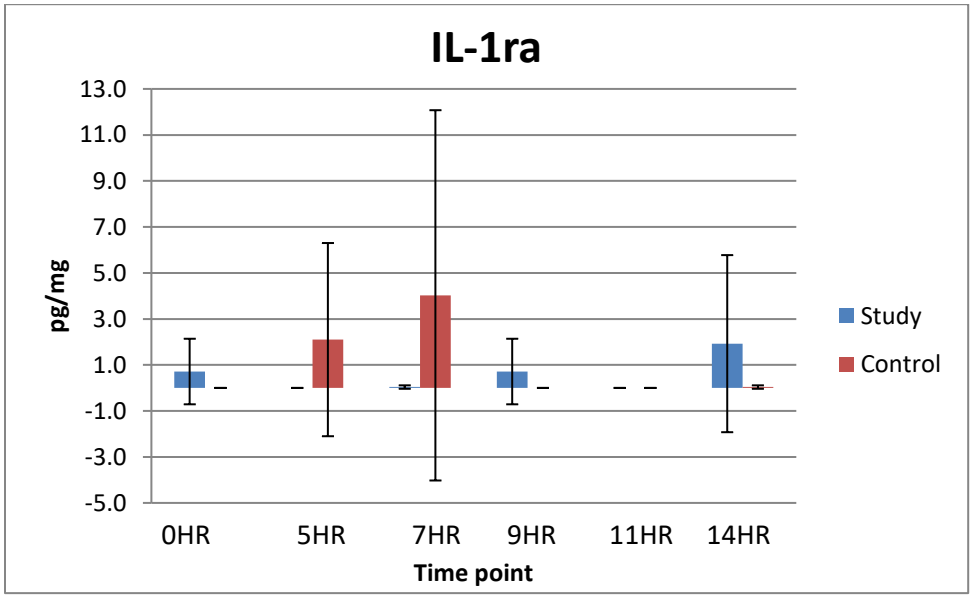
11HR
14HR
Time point
Study
Control

4. IL-1ra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3.0
1.0
-1.0
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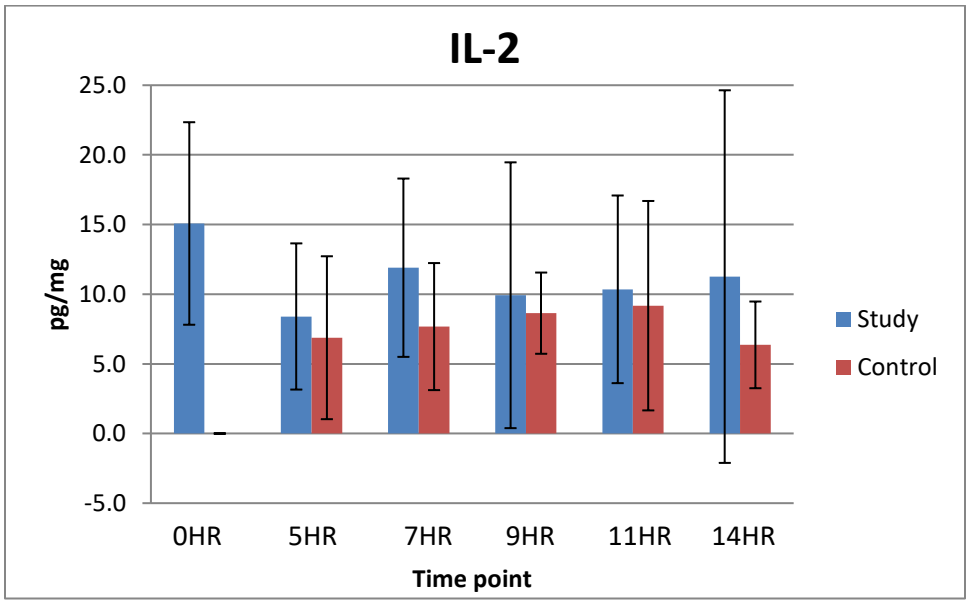
11HR
14HR
Time point
Study
Control

5. IL-2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pg/mg
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5HR
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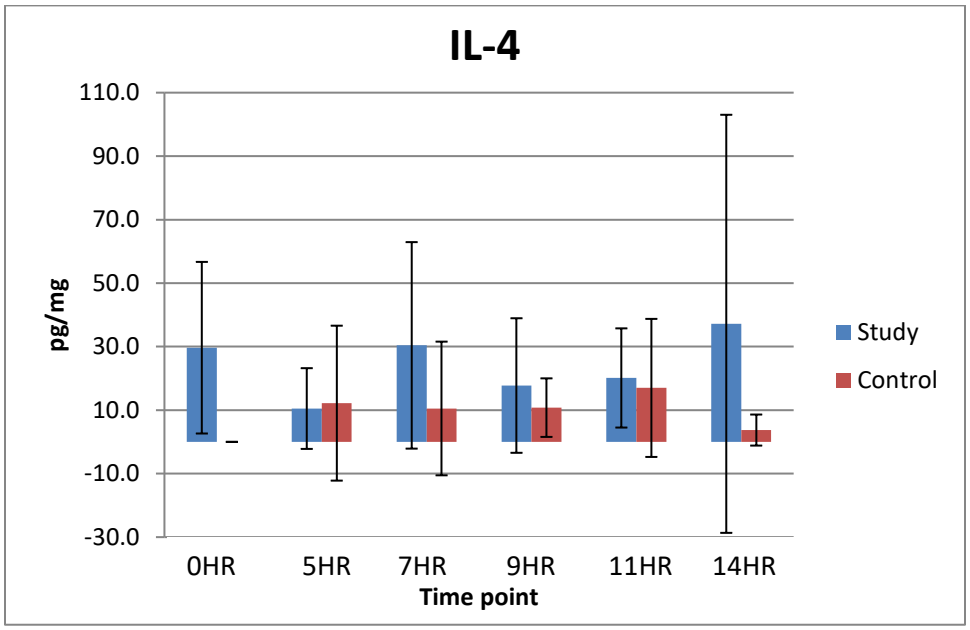
14HR
Time point
Study
Control

6. IL-4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Time point
Study
Control

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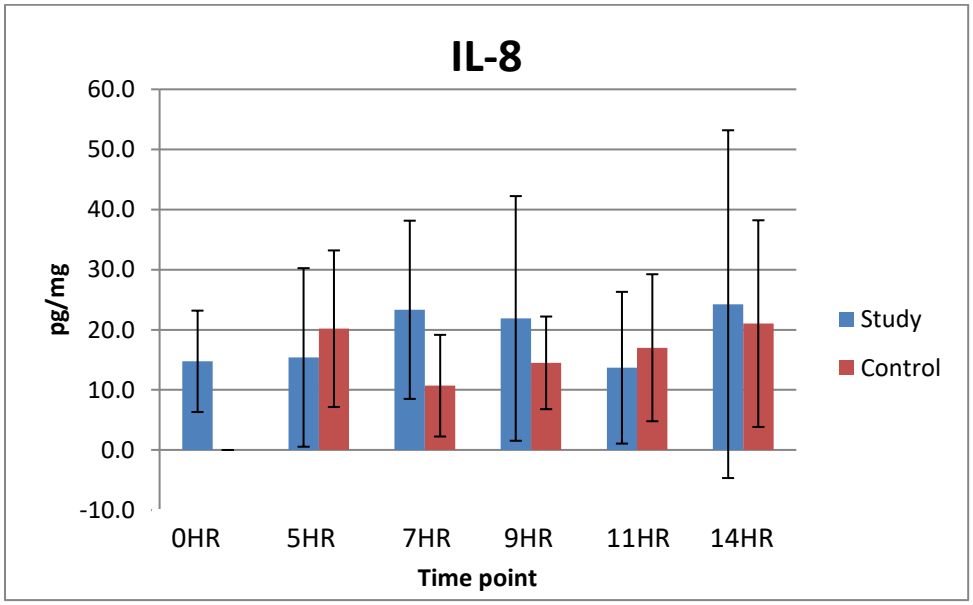
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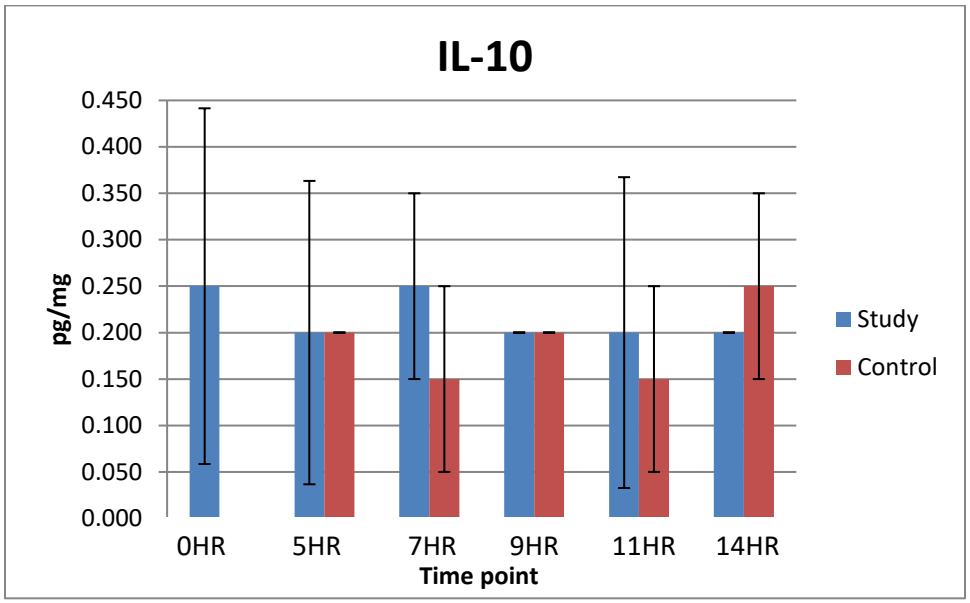
14HR
Time point
Study
Control

8. IL-10


IL-10
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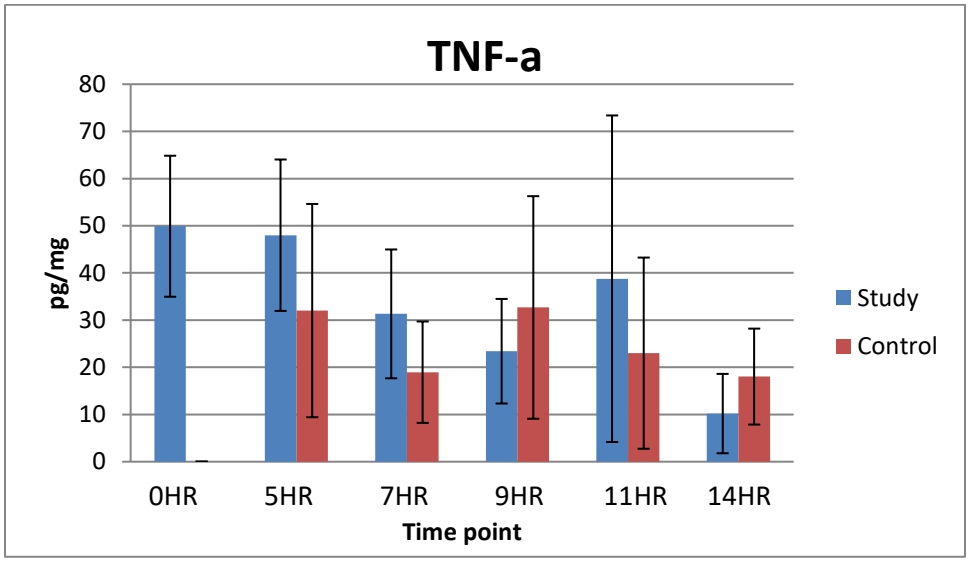
Time point
Study
Control

9. TNF-α


TNF-a
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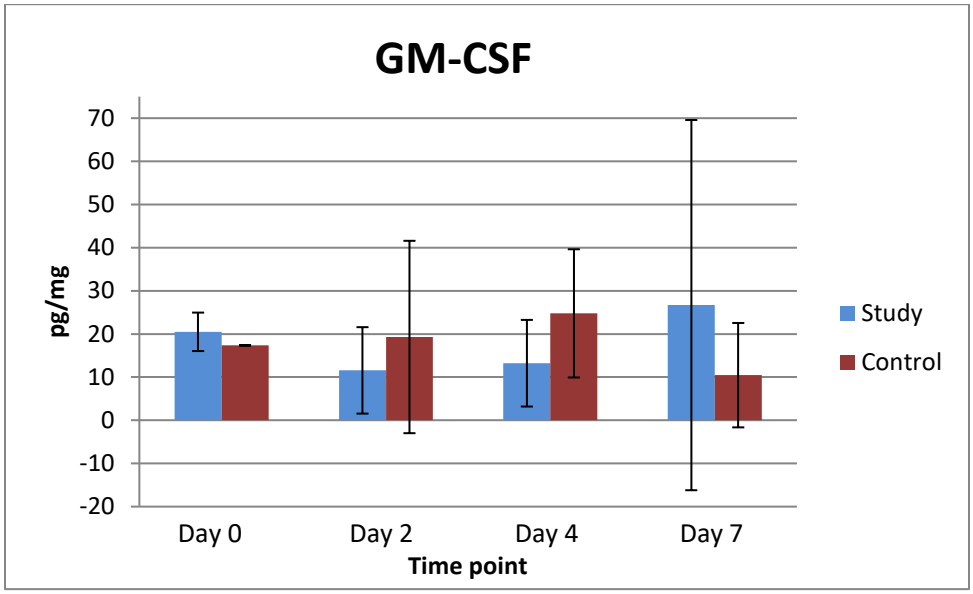
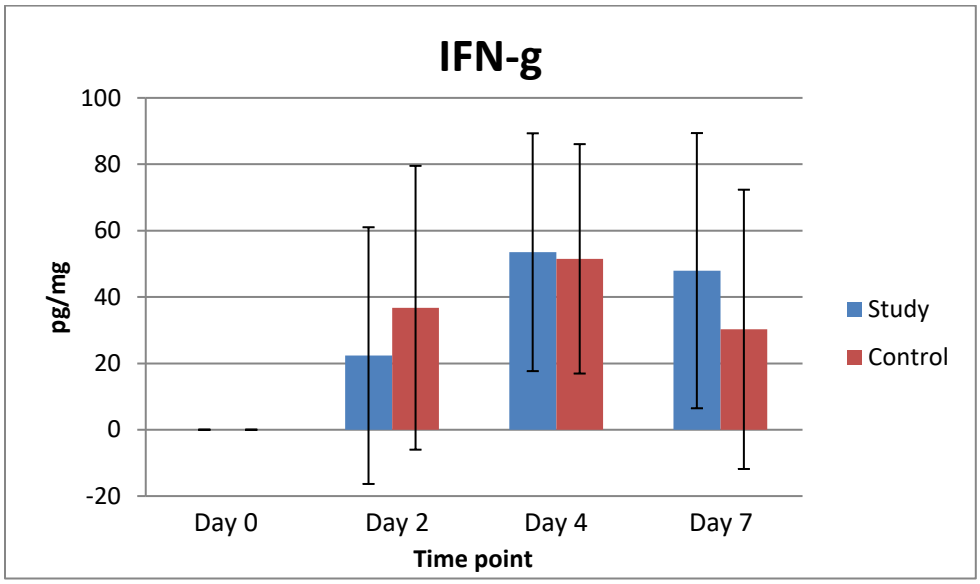
70
60
50
40
30
20
10
0
pg/mg
0HR
5HR
7HR
9HR
11HR
14HR
Time point
Study
Control

The results during the in-vivo stage (after transplantation) were the following:

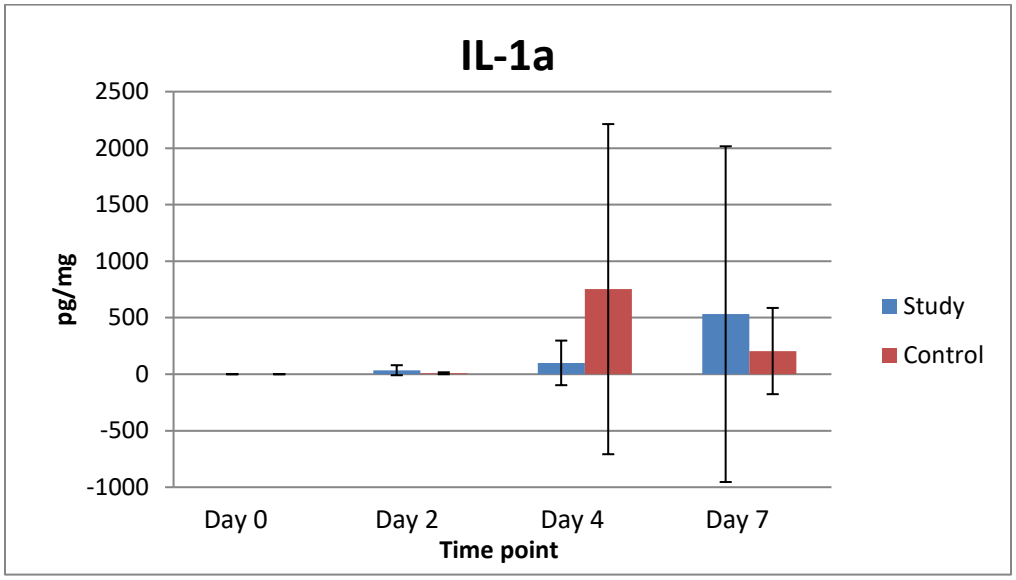
1. GM-CSF



2. IFN-g



3. IL-1a


IL-1a
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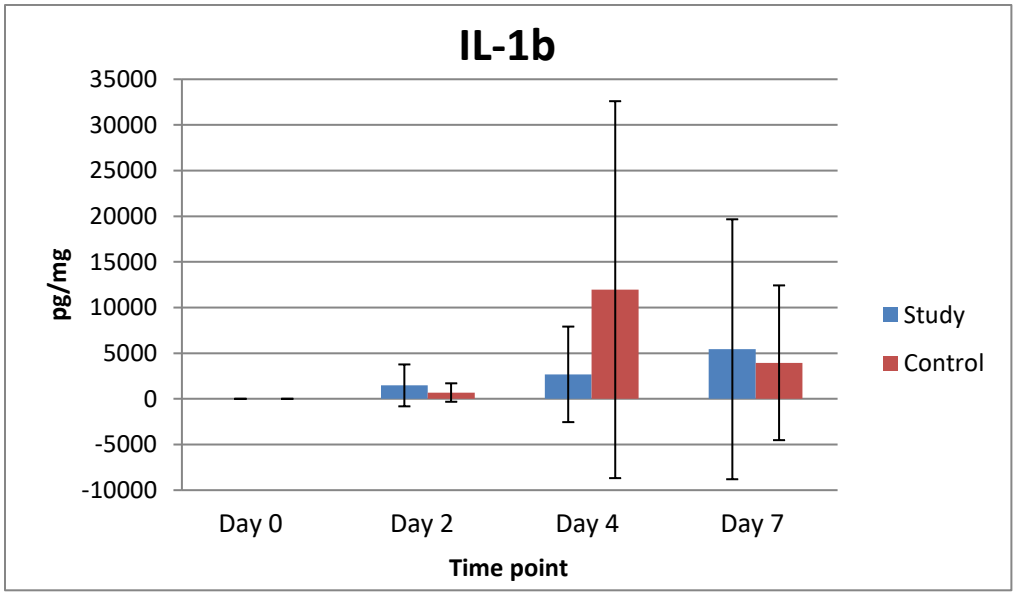
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

4. Il-1b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pg/mg
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Day 2
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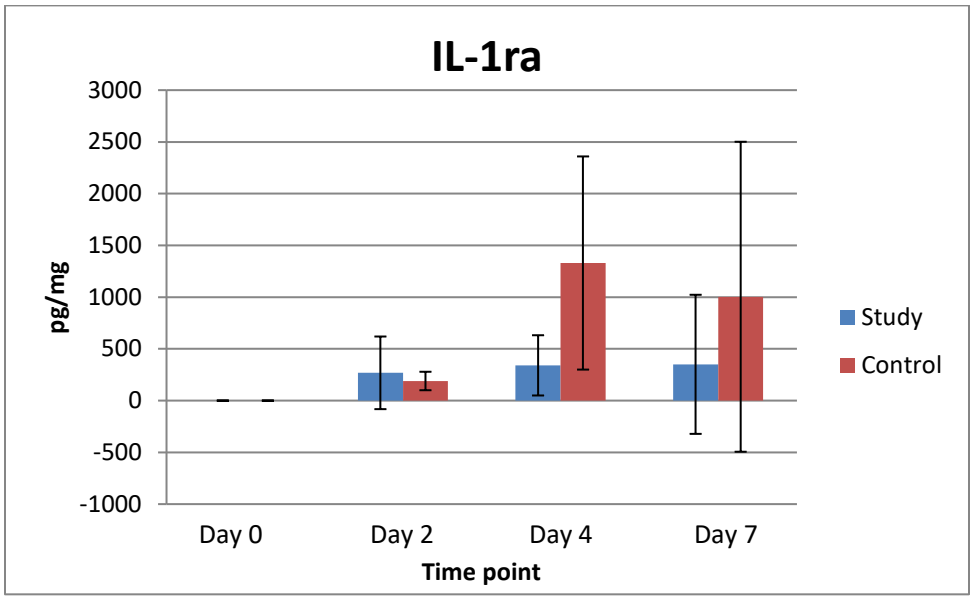
Day 7
Time point
Study
Control

5. IL-1ra


IL-1ra
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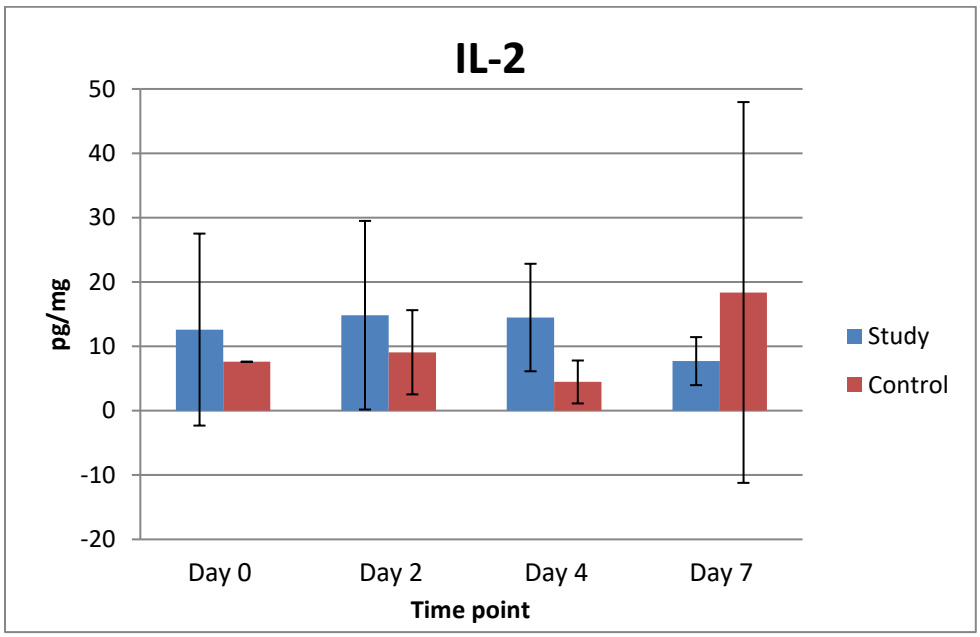
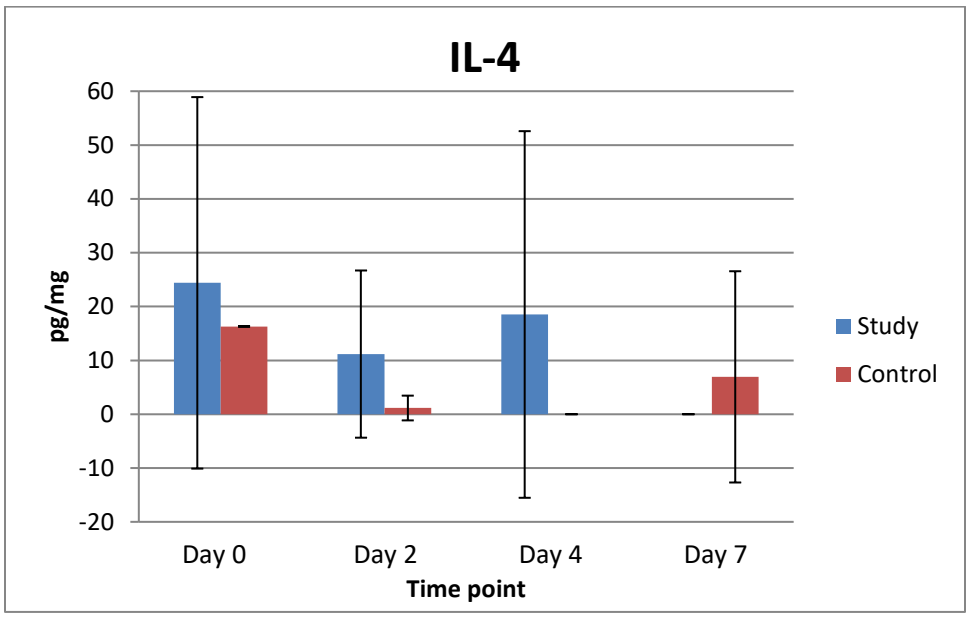
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

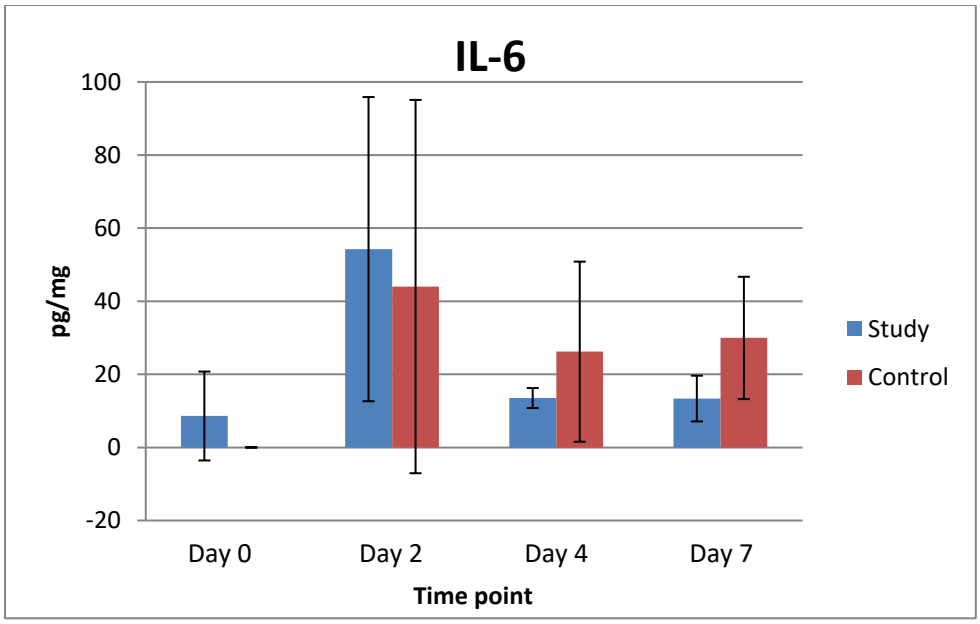
6. IL-2


IL-2
50
40
30
20
10
0
-10
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

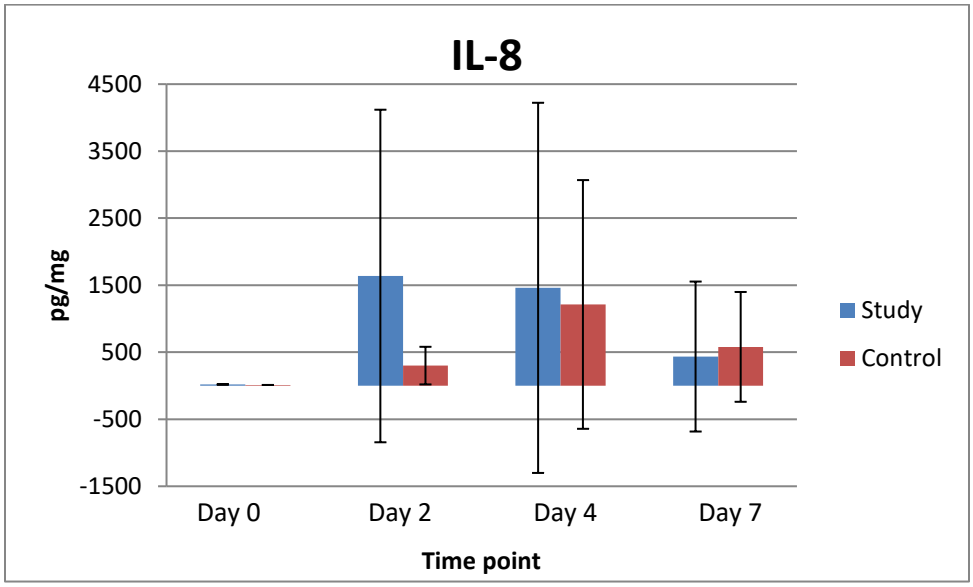
7. IL-4


IL-4
60
50
40
30
20
10
0
-10
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

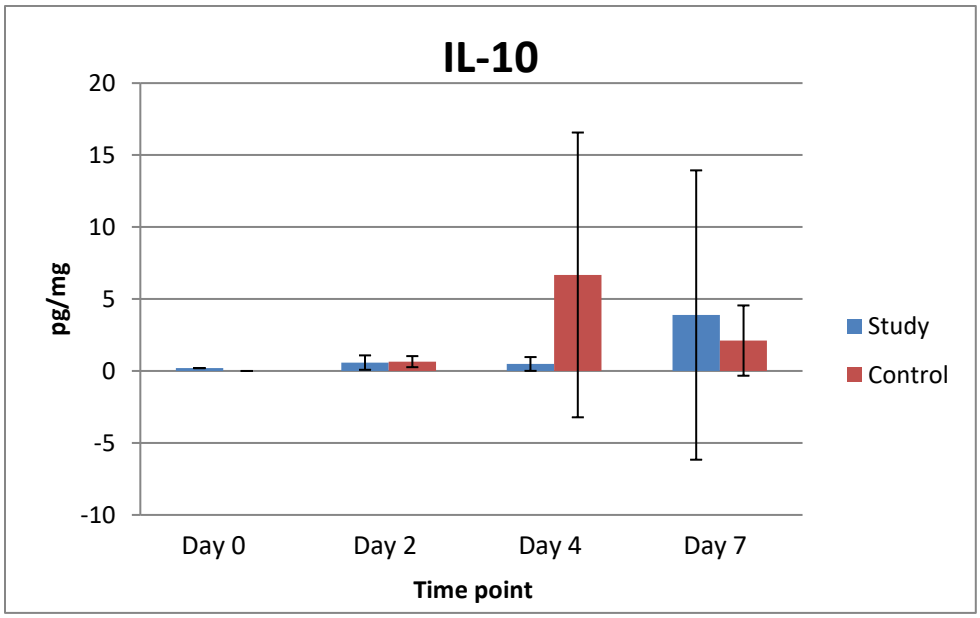
8. IL-6


IL-6
100
80
60
40
20
0
-20
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

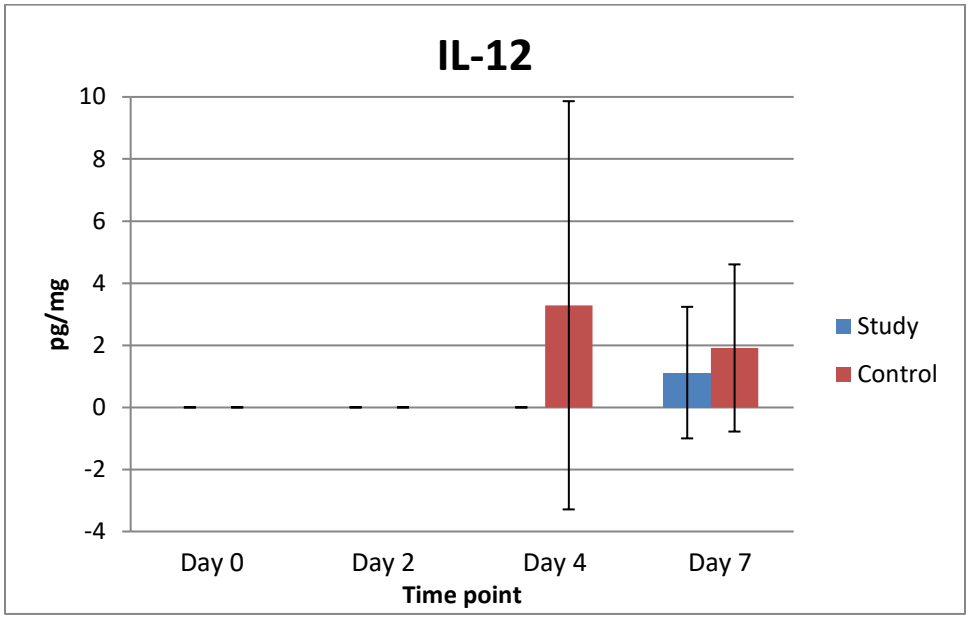
9. IL-8


IL-8
4500
3500
2500
1500
500
-500
-1500
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

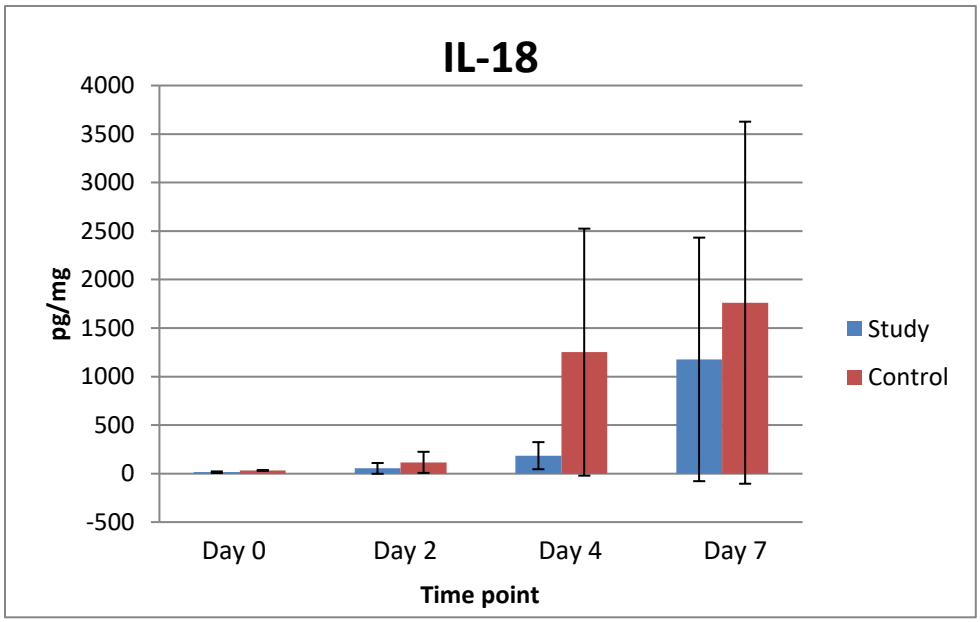
10. IL-10


IL-10
20
15
10
5
0
-5
-10
pg/mg
Day 0
Day 2
Day 4
Day 7
Time point
Study
Control

11. IL-12



12. IL-18



Clinical results

The transplant recipients received triple immunosuppressive (IS) therapy (Tacrolimus, Mycophenolate Mophetyl and Prednisone) after a single dose of Solumedrol (1g) was given intraoperatively prior to graft reperfusion. A central venous access (Broviac

catheter) was placed at the end of the surgical procedure to assure continuous venous access throughout the entire post-operative period.

The VRAM grafts were inspected daily and punch biopsies were performed on days 2, 4 and 7. The end-study necropsy was conducted on post-operative day 7 after the animals have been properly euthanized.

Clinical assessment of the VRAM grafts revealed normal features in the recipients of the MP group. The CSP animals showed signs of progressive ischemic changes (e.g. shallow ulcerations) within the graft. These shallow ulcers evolved into full ulceration of the nipples by day 7 (See figure 12).

There were no technical complications from all the surgical procedures and the animals tolerated the IS therapy well with no major adverse events.

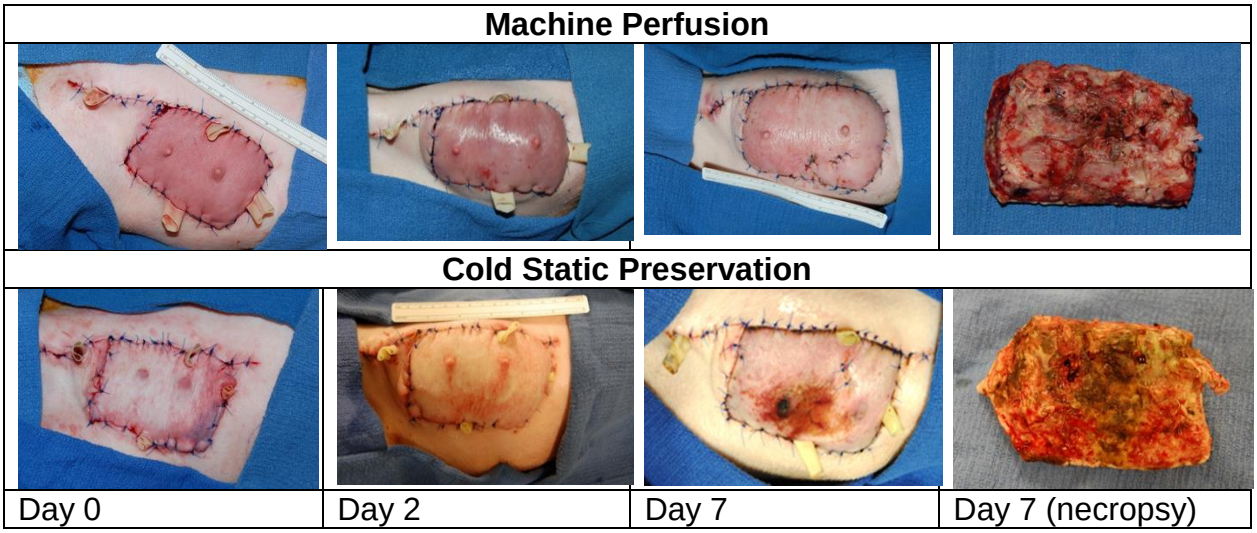


Figure 12

Myoglobin levels in the recipient's peripheral blood after transplantation

In order to assess the IRI and the level of VRAM damage after 14 hours of preservation on both groups, myoglobin levels were measured daily over a 7 day period. Figure 13 displays the data collected over a 7 days period on both groups of animals (CSP and MP).

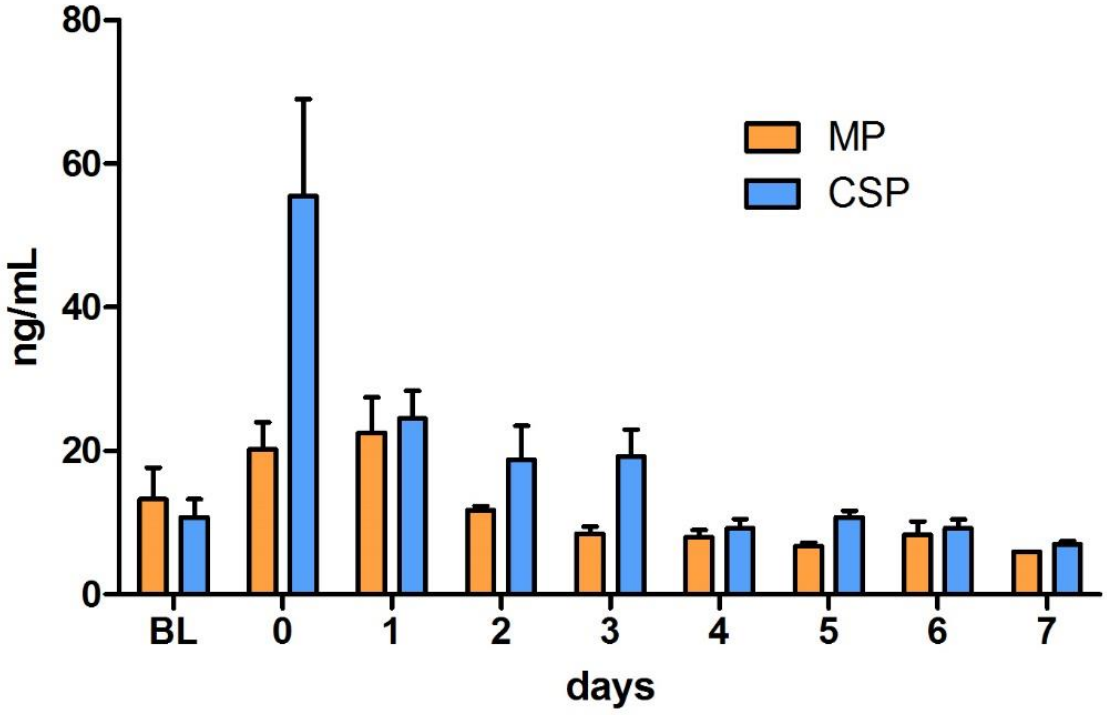


Figure 13
Histological analysis and scores for IRIs

*** p < 0.05 vs machine perfusion**
 # p < 0.05 vs Baseline (BL)
n=4 each group

A transplant pathologist who was not involved with the initial experiment design and further data collection performed a blind review of all the tissues biopsied throughout all phases of both protocols.

The following criteria were developed for a new IRI score, which has initially based on previous literature [8-10].

The VRAM grafts were divided in 5 segments:

1. Skin and epidermis
2. Subcutaneous tissue
3. Muscular tissue
4. Large vessels (hilar structures of the VRAM graft)
5. Nerve tissue

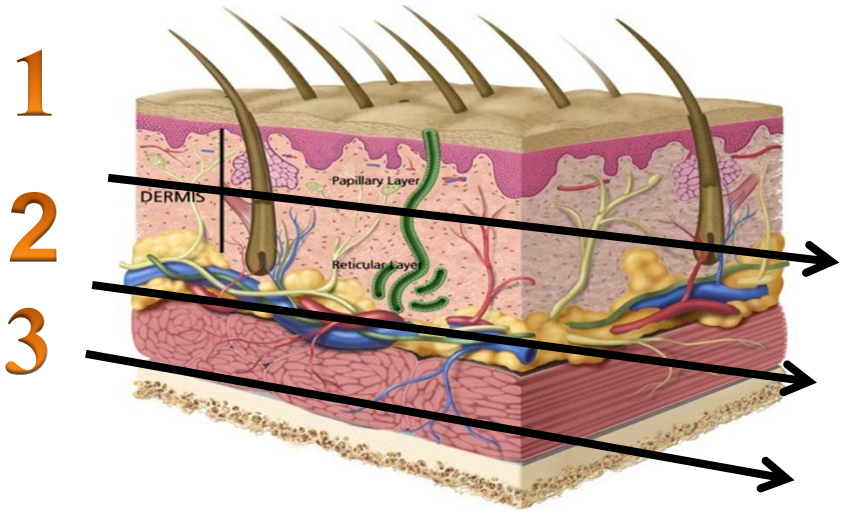


Figure 14

The following parameters were considered:

1. Tissue ischemia (e.g. contracting bands in the muscle)
2. Tissue necrosis
3. Degree of inflammation
    a. Margination of leukocytes, neutrophils and macrophages within interstitial capillaries
    b. Perivascular lymphocytic inflammation in small size vessels

A Histologic Injury Severity Score (HISS) was developed. The histological parameters analyzed within the different segments were the following:

1. Skin
    a. eosinophilic infiltration
    b. lymphocytic infiltration
    c. pyknosiskaryorrhexis
    d. karyolysis
    e. necrosis/apoptosis
2. Subcutaneous tissue
    a. perivascular infiltration
    b. lymphocytic infiltration
    c. adipocyte necrosis/apoptosis
    d. calcifications, fat necrosis
3. Muscle tissue
    a. Contraction bands (disturbed/lost cross striations)
    b. disrupted muscle fibers
    c. decomposed endomysium
    d. decomposed epimysium
    e. edema
4. Nerves
    a. intramyelinic edema
    b. endoneural edema
    c. compression induced myelin damage

    d. axonal vacuolization
    e. axotomy
5. Large hilar vessels
    a. intraluminal thrombi
    b. loss of endothelia layer small vessels
    c. loss of endothelia layer medium vessels
    d. fragmentation of internal lamina elastic
    e. fragmentation of external lamina elastic
    f. vasa vasorum involvement
    g. perivascular edema
    h. erythrocyte extravasation
    i. leukocyte adhesion
    j. leukocyte infiltration

The severity of the IRIs was scored according to the following system:

Severity of the IRI	**Score**
None	0
Mild	1
Moderate	2
Severe	3

The nerve tissue was also assessed in spite of the lack of nerve reconstruction within the VRAM graft. The assessment was mostly focused on the degree of perineural inflammation.

The hilar vessels were initially examined (gross pathology) during the end-study necropsy to rule our thrombosis stemming from potential surgical complications.
The vessels were further fixed in paraffin and sectioned for subsequent microscopic analysis.

Table 2 displays the histological classification utilized to score the IR lesions seem after reperfusion.

Score for histopathologic alterations of H&E stained sections (ref 1)	
compartment 1) Skin= epidermis + papill - retic derma	
0	tissue not affected
1	mild perivascular inflammatory infiltrate of dermal microvasculature including margination of PMN. No involvement of overlying epidermis
2	moderate perivascular Lymphocy inflammation + margination of PMN +interface inflammation with infiltration of epidermis & epithelial cell necrosis
3	severe, dense perivascular and dermal inflammation with necrosis and epiderm loss
Compartment 2: Subcutis = adipose tissue with small-medium size vessels	
0	tissue not affected
1	mild perivascular inflammatory infiltrate small size vessels including margination of PMN
2	moderate perivascular inflammation with focal adipose tissue necrosis
3	major necrosis, loss of architecture, calcinosis
comparment 3: Muscle (myocytes & intramuscle interstitial capillaries/small size vessels)	
MYOCYTES	
0	tissue not affected
1	mild ischemic changes (bands of contraction // loss of striation (and or mild, localized perivascular inflammatory infiltrate less than 50%
2	multifocal ischemic changes/necrosis and/or perivascular inflammatory infiltrate including margination of leukocytes (neutroph and macrophages) in interstitial capillaries
3	major muscle cell necrosis and calcification
interstitial inflammation /microvasculature	
0	normal
1	mild interstitial/ perivascular inflammatory infiltrate including margination of PMN.
2	moderate interstitial/perivascular Lymphocy inflammation including margination of PMN
3	severe, dense interstitial/perivascular inflammation
Nerves Histological evaluation showed non specific changes - need additional studies	
0	tissue not affected
1	mild perineural inflammatory infiltrate, mild vacuolization and mucoid degeneration
2	severe perineural inflammatory infiltrate, vacuolization and mucoid degeneration, minor alterations of nerve structure
3	severe degeneration and loss of typical nerve structure
Vessels medium and large size: Pedicle	
0	tissue not affected
1	reactive endothelial cells
2	perivascular infiltration and/or alterations in vessels architecture (lamina elastica, smooth muscle)
3	major vessel damage, loss of architecture/necrosis/ intraluminal thrombi
	Histopathologic alteration score

Table 2

Results – histological analysis

The skin segments from both groups had only mild changes during the 14 hour preservation period. There were no signs of edema and/or endothelial cell damage in the VRAM grafts preserved with MP. Figure 15 displays 2 panels of serial biopsies performed in the VRAM grafts' skin during preservation. There is a mild leukocyte infiltration at the dermis and no signs of additional tissue damage during this initial stage.

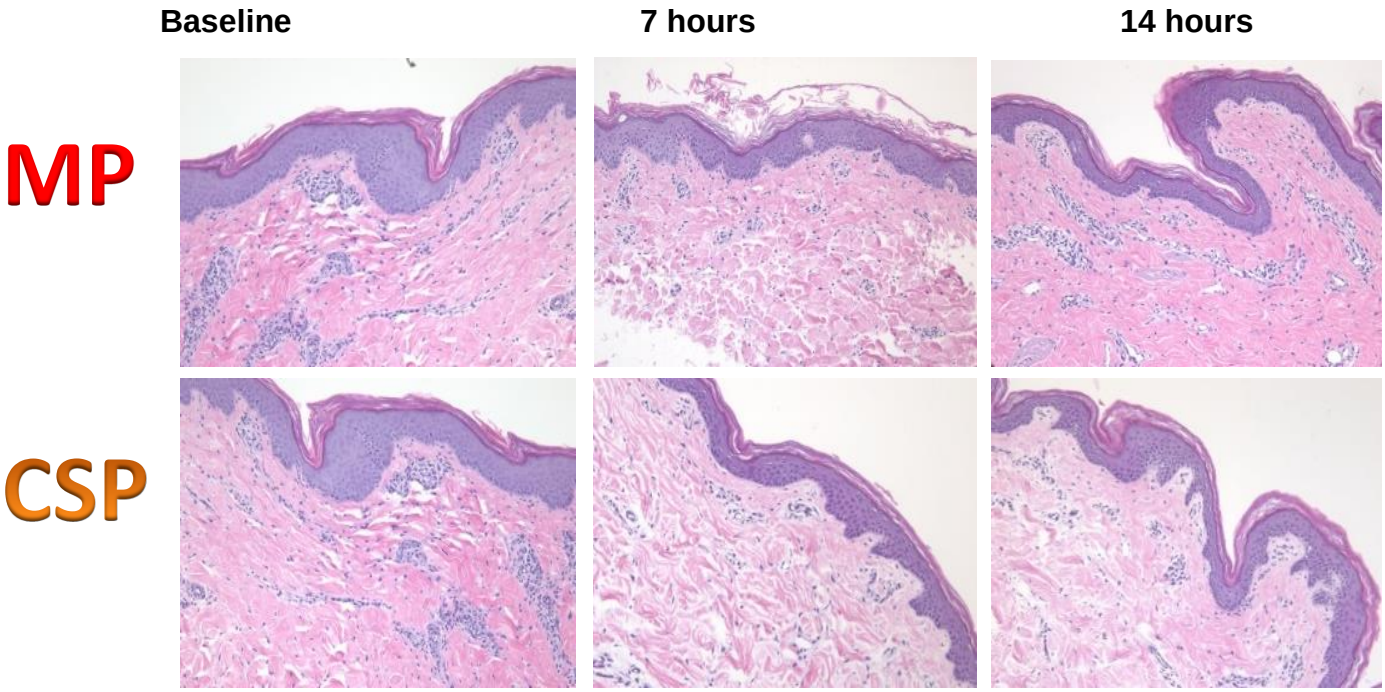


Figure 15

Interesting enough, the muscle segments showed early signs of tissue damage (contraction bands) when the CSP group was compared to the MP group.

The development of hypercontracture and identifiable contraction bands on histology has been extensively studied in the myocardium as a clear and well established sign of early ischemia in striated muscle.

This phenomenon has been previously described [14] and summarized on Figure 16 , where the persistent lack of oxygenation would be the main factor involved in the pathophysiology of this anatomical feature.

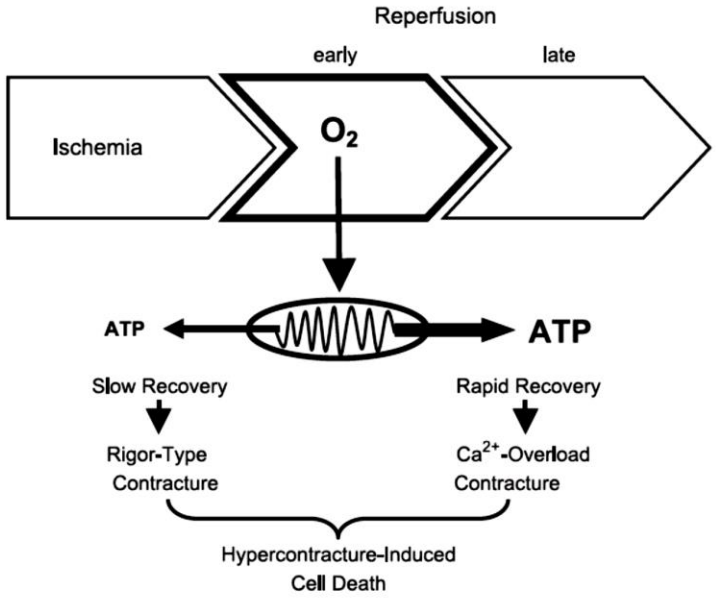
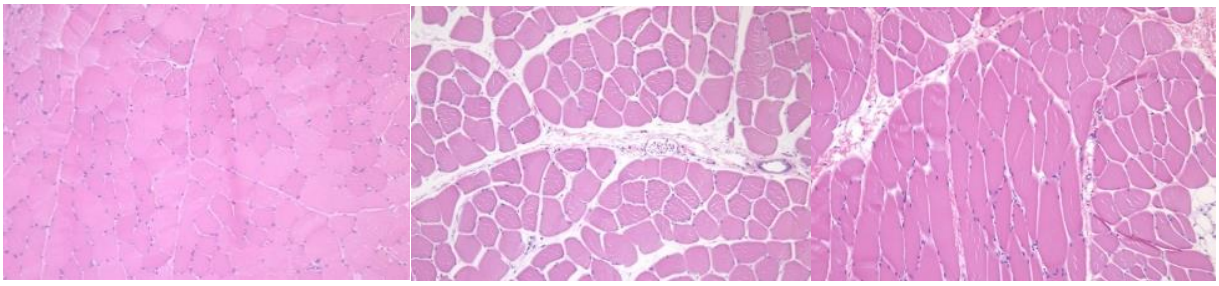


Figure 16

Figure 17 displays panels of serial biopsies in both groups during the 14 hour period for graft preservation. The MP group shows no major histological changes. The CSP group shows early formation of contraction bands within 7 hours and further expansion within 14 hours.

MP Baseline MP 7 hours MP 14 hours



CSP Baseline CSP 7 hours CSP 14 hours

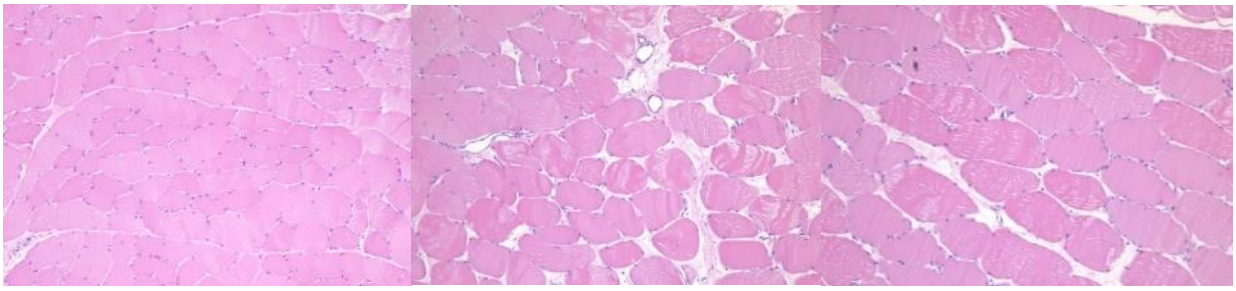


Figure 17

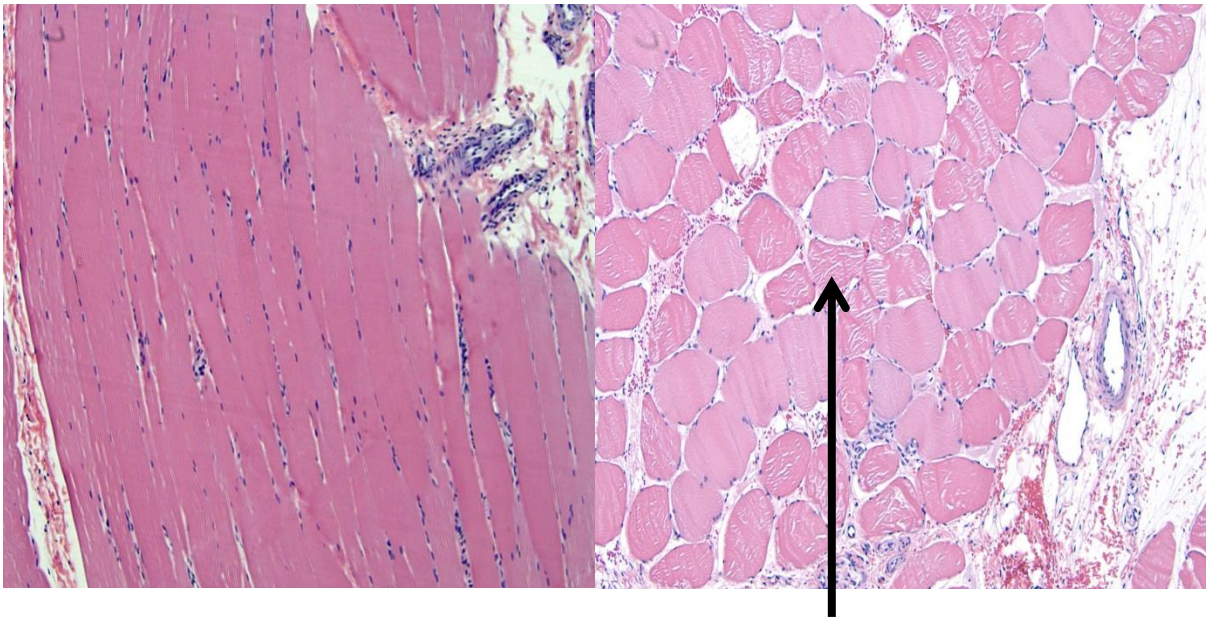
Post-transplantation period

The VRAM grafts were biopsied on days 2, 4 and 7. Early post-reperfusion events were assessed on day 2. It has been clearly demonstrated that the magnitude of the IRIs were more pronounced in the CSP group.

The CSP group, in analogy to the myocardium during ischemic events, showed persistent signs of contraction bands, which became diffuse after VRAM graft reperfusion. There were no histological changes in the MP group on day 2, showing the benefits of the effective oxygenation provided by the MP/HBOC system.

Figure 18 displays a comparison between both groups on the 2nd post-operative day (POD), where the contraction bands initially seem focally at 7 hours in the CSP groups evolved to a diffuse pattern within the muscle tissue.

MP **CSP**



Normal skeletal muscle Contraction bands

Figure 18

The histological features between the two groups became much more pronounced on the 4th and 7th POD (See Figure 19). The MP group VRAM grafts showed resolution of the mild inflammatory process stemming from none to mild IRIs. The CSP group showed progression of the moderate to severe IRI lesions seen after reperfusion, which were characterized as moderate myocytes ischemic changes and mild interstitial inflammation. The CSP on day 4 displayed features of disseminated degeneration of muscle with diffuse contraction bands. The lack of oxygenation in the CSP resulted in permanent mitochondrial damage and propagation of the Ca^{2+} overload induced contracture leading into further myocyte necrosis.

MP – 4th POD CSP – 4th POD

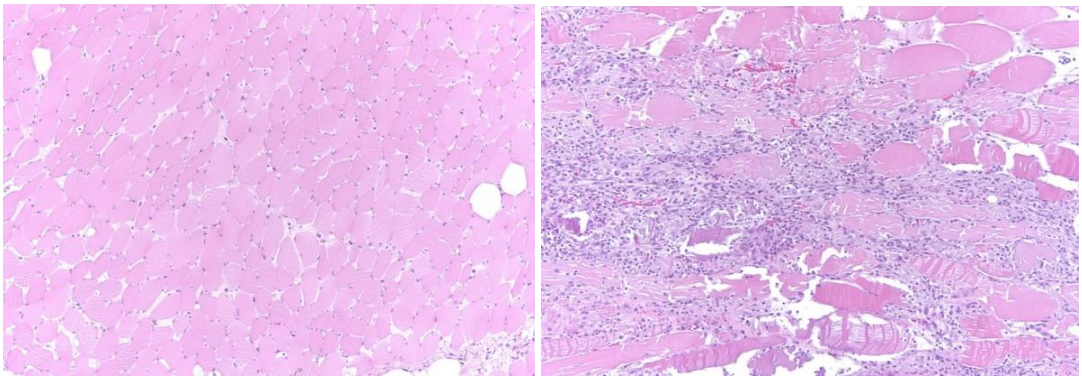


Figure 19

The MP group showed a very mild inflammatory infiltrate within the muscular tissue. The CSP group showed a moderate to severe inflammatory infiltrate composed mainly by lymphocytes and neutrophils. There was exacerbation of the contraction bands and diffuse interstitial edema. Diffuse apoptosis and early necrosis were also seen in the CSP group.

The histological findings on the 7th POD were even more pronounced when the two groups were compared (See Figure 20). The MP group displayed a limited interstitial infiltrate and mild interstitial edema. The CSP group displayed a severe inflammatory infiltrate composed mainly by neutrophils, macrophages and eosinophils. There were diffuse necrosis and several segments of tissue with calcinosis, where histiocytes surrounded wide spread necrotic areas. There was very limited viable muscle tissue on the CSP group on the 7th POD. The same findings were further confirmed by the end-study necropsy.

MP – 7th POD CSP – 7th POD

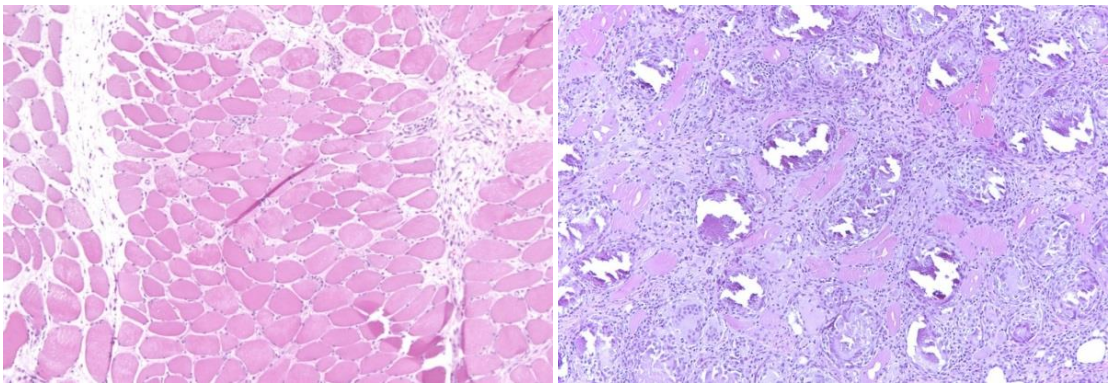


Figure 20

Overall HISS

The HISS were calculated by the combining (mean±SD) the values obtained in each time point (PR= post-reperfusion, 2= 2nd POD, 4= 4th POD and 7=7th POD) of all 3 segments of the VRAM grafts.

Figure 21 displays the HISS scores over the duration of the experiments. The MP group had significantly lower scores across all time points.

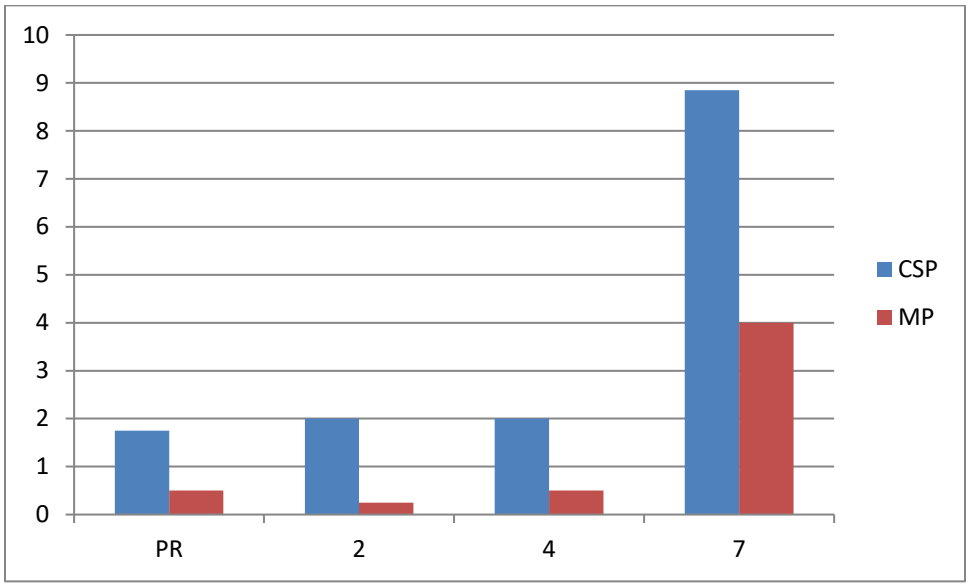


Figure 21

Metabolomics

Tissue samples from the VRAM grafts were obtained during preservation (0, 5, 9 and 14 hours) and after the transplant procedure on POD 0, 2, 4 and 7. These tissues were immediately frozen (OCT) and further submitted to Metabolon Inc., Raleigh, NC for metabolomics analysis. Following receipt, samples were inventoried, and immediately stored at -80ºC. At the time of analysis samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass

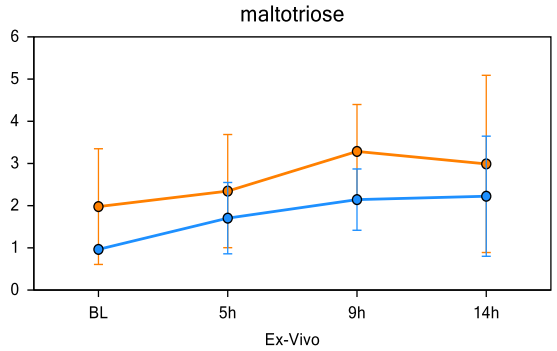
spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion.

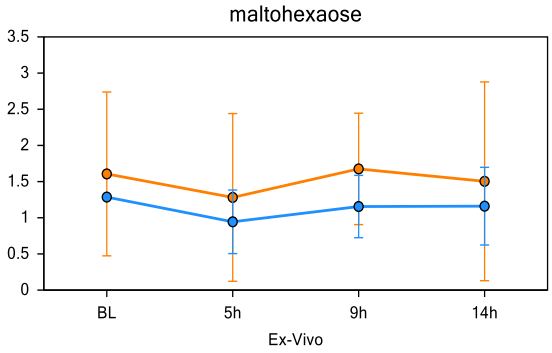
Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples.

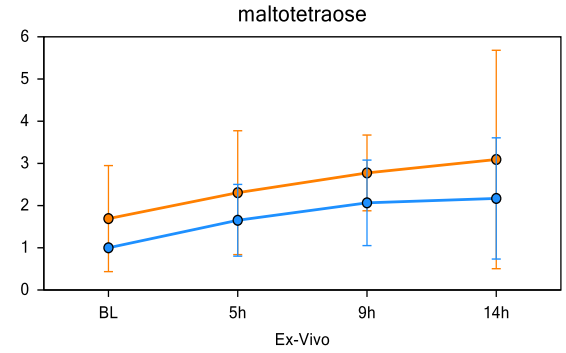
There were 653 compounds analyzed. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch's two-sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (*q*-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. The *q*-value describes the false discovery rate; a low *q*-value ($q<0.10$) is an indication of high confidence in a result. While a higher *q*-value indicates diminished confidence, it does not necessarily rule out the significance of a result.

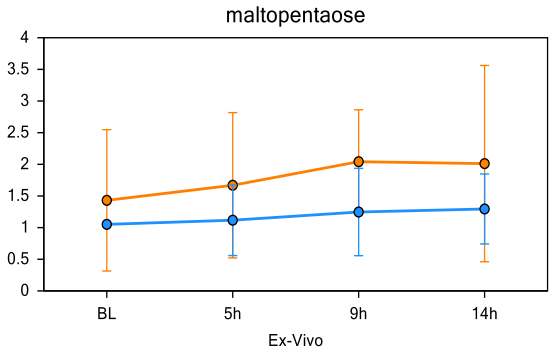
Results

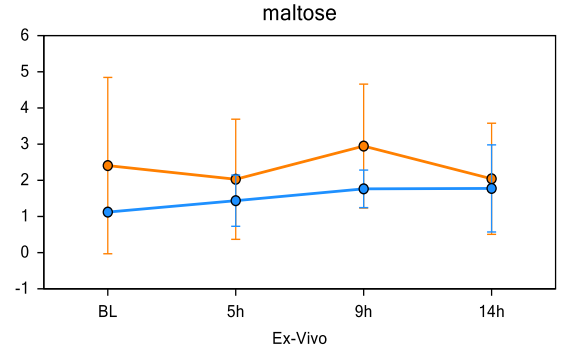
The VRAM grafts in the MP system sustained an intact energetic metabolism fueled by glucose over the 14 hour period of preservation when compared to the CSP group. Glycogen reserves were higher in the MP group. There was adequate glucose supply in the MP and no signs on glycogen breakdown. The graphics below represent metabolites levels during preservation. The MP group is represented in yellow (–) and the CSP group is represented in blue (–).



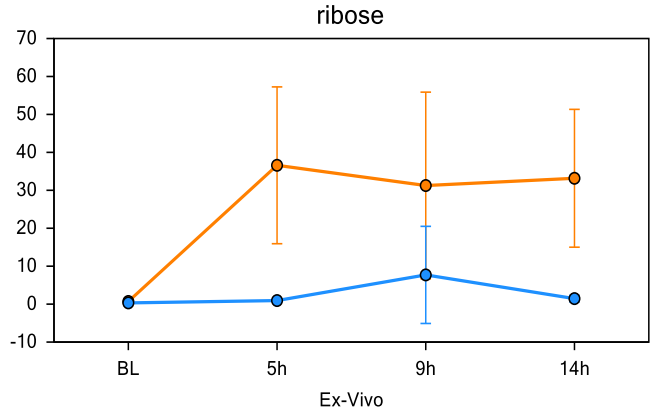

maltohexaose
3.5
3
2.5
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo

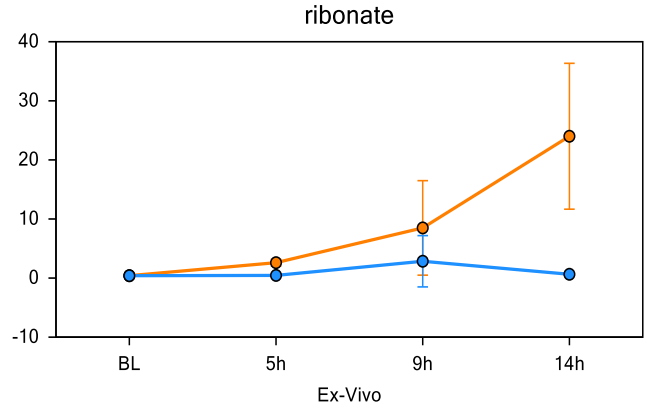

maltotetraose
6
5
4
3
2
1
0
BL
5h
9h
14h
Ex-Vivo

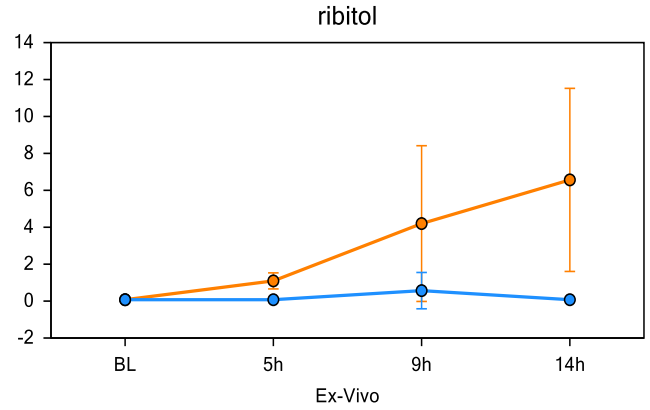

maltopentaose
4
3.5
3
2.5
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo


maltose
6
5
4
3
2
1
0
-1
BL
5h
9h
14h
Ex-Vivo

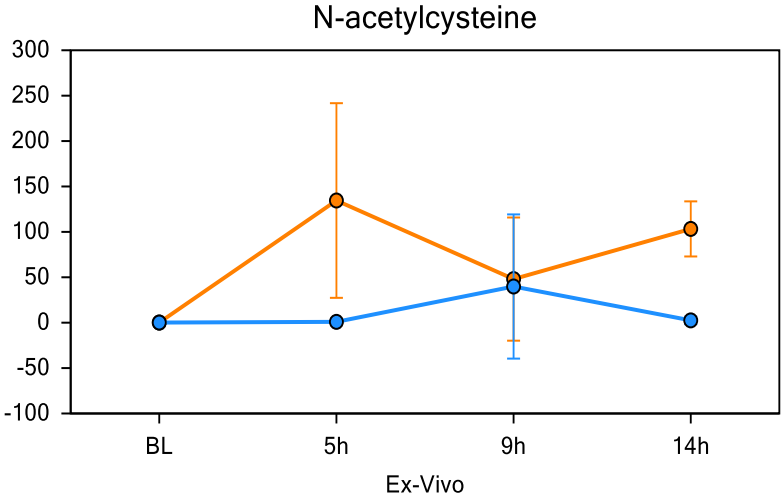
The pentose metabolites were significantly higher in the MP group, showing a higher anabolic state when compared to the CSP group. The CSP group appeared to have a sustained catabolic state when compared to the MP group. There were signs of higher production of nucleotides and nucleic acids precursors in the MP. As previously seen in our experience with liver allografts under the MP/HBOC system, there was a significant (30 fold higher) increase in the metabolic pathways related to cell regeneration once oxygenation was effectively provided ex-vivo during preservation. There were signs of higher production of aromatic amino acids in the MP group when compared to the CSP group.

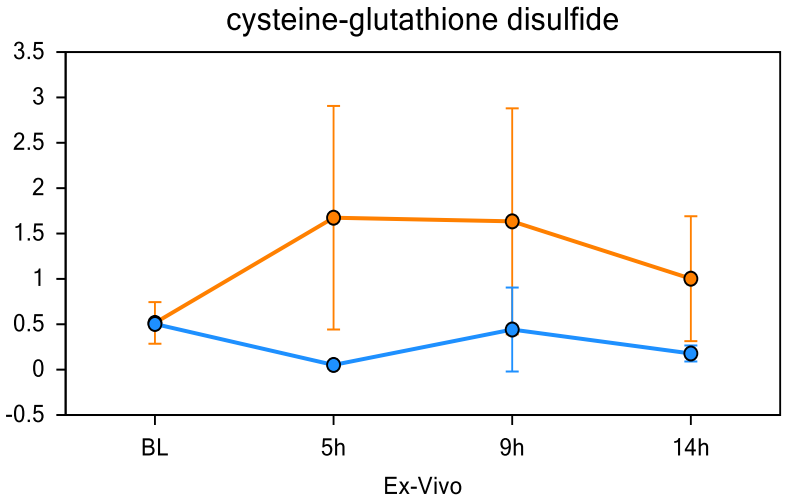




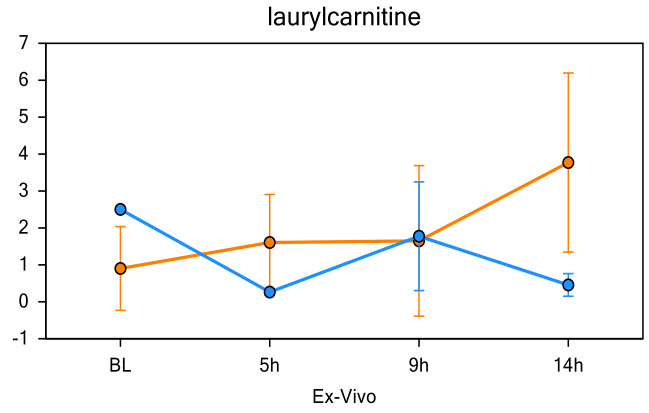


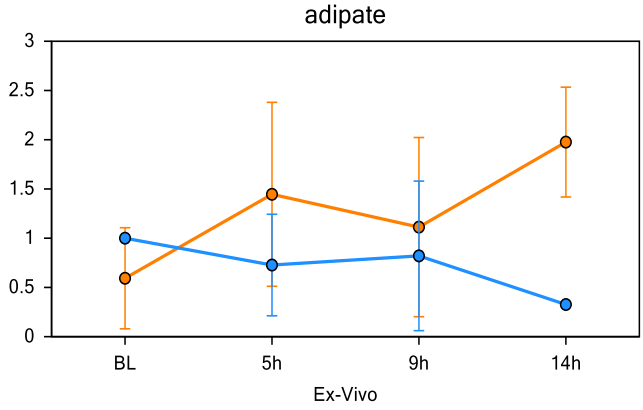
The MP/HBOC system provided more effective anti-oxidant pathways when compared to the CSP group. There were higher levels of end-products from oxidized stress in the MP group, which can be seen as an indirect sign of lower stress from less significant IRIs when compared to the CSP group.

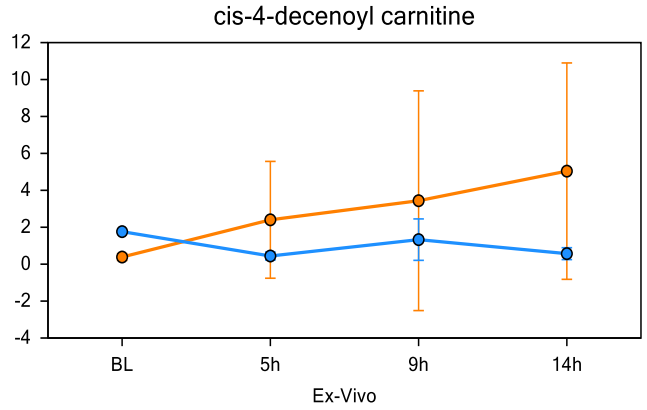


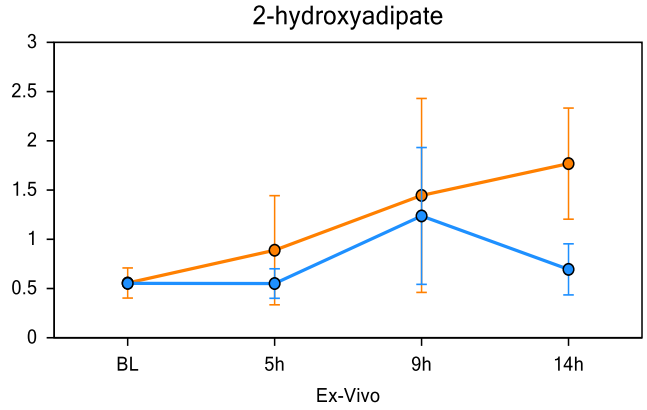


Contrary to our previous experience with livers, the VRAM grafts under the MP/HBOC system showed lower fatty acid β-oxidation when compared to the CSP group. This means a lower amount of fatty acids into the mitochondria as a source of fuel. This also favors our initial findings regarding the preferential pathway for glucose as the primary source of energy in striated muscles.



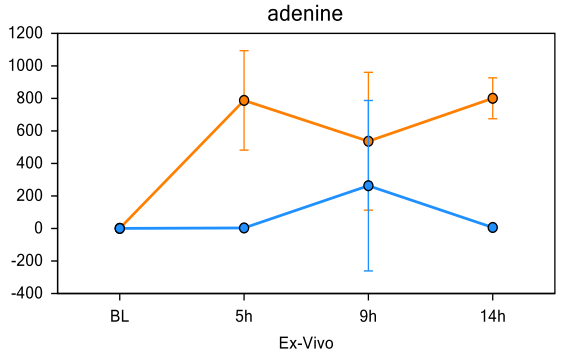


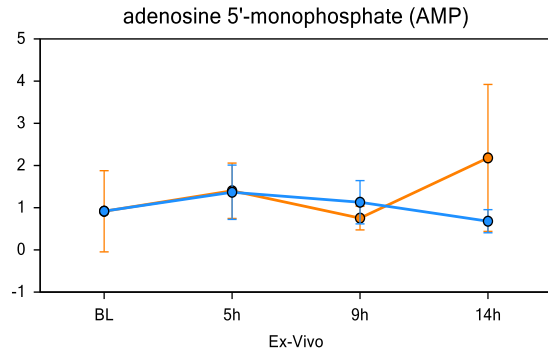


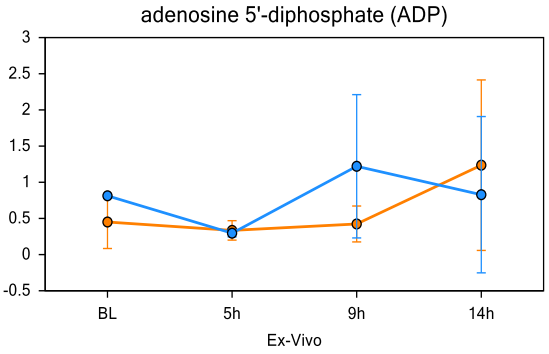


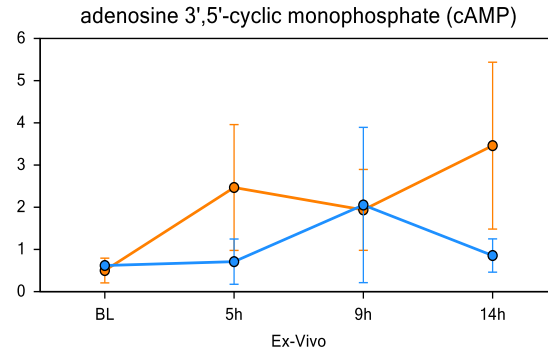
Further analysis of the purine metabolism (adenine components) showed indirect signs of higher ATP production in the MP group when compared to the CSP. The adenine family has a variety of roles in cellular respiration and protein synthesis.

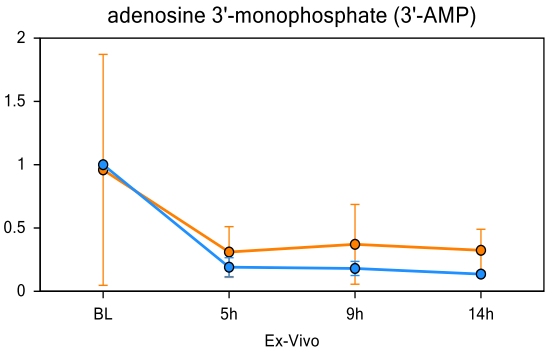
There were higher levels of cAMP in the MP group. AMP is used as a monomer in RNA synthesis. The cAMP as a derivative of ATP has a significant role in signal transduction.




adenosine 5'-monophosphate (AMP)
5
4
3
2
1
0
-1
BL
5h
9h
14h
Ex-Vivo


adenosine 5'-diphosphate (ADP)
3
2.5
2
1.5
1
0.5
0
-0.5
BL
5h
9h
14h
Ex-Vivo


adenosine 3',5'-cyclic monophosphate (cAMP)
6
5
4
3
2
1
0
BL
5h
9h
14h
Ex-Vivo


adenosine 3'-monophosphate (3'-AMP)
2
1.5
1
0.5
0
BL
5h
9h
14h
Ex-Vivo

No Cost Extension Period: Data Analysis

INTRODUCTION:

This study has been an extension of our successful proof-of-concept experiments with machine perfusion (MP) in combination with a newly developed hemoglobin based oxygen carrier (HBOC) solution under subnormothermic (21°C) conditions as a way to enhance graft preservation and immunologic outcomes.

Our pre-clinical proof-of-concept large animal experiments in preserving composite tissue allografts (CTA) over a 14 hour period prior to transplantation have shown significant benefits in minimizing ischemia reperfusion injuries when ex-vivo oxygenation was effectively provided by the MP/HBOC system. These experiments involved a combination of regular data sets from standard clinical and laboratorial assessments, in addition to extensive data collection from histology, protein-level inflammatory markers and metabolomics. The CTA were continuously sampled before, during, and after preservation (14 hours) and repeatedly over the post-operative period (7 days). Inflammatory markers and metabolomics were assessed from both the tissues and the perfusate during CTA preservation.

Previous studies with a similar MP/HBOC technique in liver preservation have shown great benefit in adding new analytical tools to assess the system-level interactions among inflammatory markers and metabolomics [1]. Our group has successfully applied Dynamic Bayesian Network (DyBN), Dynamic Network Analysis (DyNA) and Principal Component Analysis (PCA) to elucidate the complex interactions among various inflammatory markers. This innovative approach has already uncovered the role of an NLRP3 inflammasome-regulated response in liver allografts when receiving adequate ex-vivo oxygenation during preservation with our proprietary MP/HBOC system. A recent manuscript showing the benefits of these additional analytical protocols has just been recently accepted for publication [2].

The aim of this extension study was to apply a similar approach to our CTA data generated under our most recent DOD award (Ex-Vivo Machine Perfusion with Novel Oxygen Carrier System to Enhance graft Preservation and Immunologic Outcomes - W81XWH-13-2-0061, 09/2013 – 09/2015), where a system-based and more extensive data analysis would reveal the dynamics of potential horizontal and vertical interactions among regulatory pathways experienced by the inflammatory markers previously evaluated by standard statistical analysis.

The main objectives were the following:
1. Elucidate the intrinsic dynamic interactions occurring overtime among all the inflammatory previously assessed in our original study.
2. Discover potential inflammatory patterns that would further differentiate the biological behavior of the 2 groups (MP and control) since the clinical, histological and

metabolomics data has shown significant benefits in the MP, where effective ex-vivo oxygenation has been provided during CTA preservation (14 hours).
3. Enhance the analytical process for inflammatory markers in MP experiments, since our previous experience with livers has proven to be very effective in revealing additional biological interactions initially missed by standard statistical analysis.

3. Key words

Machine perfusion (MP), cold static preservation (CSP), cold ischemia time (CIT), hemoglobin based oxygen carrier (HBOC), composite tissue allotransplants (CTAs), subnormothermic (SN), vascularized composite allotransplantation (VCA), vertical rectus abdominis (VRAM), University of Wisconsin solution (UW), superior epigastric artery (SEA), cytokines, metabolomics, ischemia-reperfusion injury (IRI), Belzer Machine Perfusion Solution (BMPS), Dynamic Bayesian Network (DyBN), Dynamic Network Analysis (DyNA), Principal Component Analysis (PCA).

4. Specific Aims and Accomplishments

Specific Aims

- Aim 1: to define principal drivers and intrinsic regulatory networks of protein-level inflammatory mediators as related to the effect of MP /HBOC on grafts during the preservation period (ex-vivo).
- Aim 2: to define principal drivers and networks of protein-level inflammatory mediators as related to the effect of MP /HBOC on the immune profile of various flap tissues after transplantation (in-vivo).

Objectives/Accomplishments

Our objectives in this additional analytical approach were to define the principal drivers of inflammation as well as interconnected inflammatory networks in 1) graft tissue during the period of preservation with MP /HBOC, and 2) following transplantation.

4.1: Define statistically significant changes in inflammatory mediators in graft tissue during the period of preservation with MP /HBOC.

To define statistically significant differences in inflammatory mediators assayed in graft tissue during the period of preservation with MP /HBOC as a function of experimental group, we conducted standard statistical analyses of all data essentially as we described recently [3-10]. Analyses were performed using SigmaPlot™ 11 (Systat Software, Inc., San Jose, CA), (MatLab® (MathWorks, Inc., Natick, MA), and StatView® (SAS Institute, Inc., Cary, NC). Comparisons of Luminex™ and qRT-PCR gene expression data were analyzed using ANOVA and the Tukey-Kramer *post hoc* test where appropriate. When only two comparisons could be made, an unpaired two-sided t-test was used. Experimental results were determined to be statistically significant when $p < 0.05$. Continuous variables were expressed as mean ± SEM. For the ANOVA outcomes, 80% power and 5% significance were used to find a 25% difference in outcomes. We expected

to find statistically significant differences in inflammatory mediators in samples from graft tissue preserved with MP /HBOC vs. control.

4.2: Define principal drivers of inflammation in the VRAM graft tissue during the ex-vivo preservation period with MP /HBOC.

To define principal drivers of inflammation in graft tissue during the preservation period with the MP /HBOC system as a function of experimental group, we carried out PCA of all data essentially as we described recently . The goal of this analysis was to identify the subsets of inflammatory mediators (in the form of orthogonal normalized linear combinations of the original mediator variables, called principal components) that were most strongly correlated with a given experimental procedure or outcome, and that thereby could be considered principal drivers of each response. To perform this analysis, the data was first normalized for each mediator (i.e. a given value divided by the maximum value for a given inflammatory mediator), so that all mediator levels could be converted into the same scale (from 0 to 1). In this way, any artefactual effects on variance due to the different ranges of concentration observed for different cytokines were eliminated. Only sufficient components to capture at least either 70% or 95% of the variance in the data were considered. From these leading principal components, the coefficient (weight) associated with each cytokine was multiplied by the eigenvalue associated with that principal component. This product represented the contribution of a given inflammatory mediator to the variance accounts for in that principal component. The overall score given to each cytokine/chemokine was the sum of its scores in each component. This gave us a measure of a cytokine's contribution to the overall variance of the system. We expected to find different inflammatory drivers in graft tissue preserved with MP /HBOC vs. control.

4.3: Define the dynamic networks of inflammation in the VRAM graft tissue during the period of preservation with MP-BMPS/HBOC.

Dynamic Bayesian Network (DyBN) inference and Dynamic Network Analysis (DyNA) were used to define networks of cytokines and chemokines model the evolution of the probabilistic dependencies within a system over time. DyBN inference were carried out using MATLAB™ (The Math Works, Inc., Natick, MA), using an algorithm adapted from Grzegorczyk & Husmeier[2] and revised recently by the Vodovotz group [11]. DyBN allowed us to assess the dominant inflammatory mediators and the probable interaction among various mediators, including possible feedback loops. DyNA is an algorithm developed by the Vodovotz group using MATLAB™; though this algorithm cannot show feedback loops, it does highlight correlations across individual time intervals, allowing for a representation of the connectivity among inflammatory mediators as a function of time [12] . We expected to find different inflammatory networks in graft tissue preserved with MP-BMPS/HBOC vs. control, since we detected significant differences between the two groups after the initial clinical, histological and metabolomics analysis

4.4: Define statistically significant changes in inflammatory mediators following VRAM implantation into the recipient (graft transplantation).

This was performed essentially as described for data on inflammatory mediators assayed in graft tissue during the period of preservation with MP /HBOC. We expected to find statistically significant differences in inflammatory mediators in samples from transplanted tissues after having been preserved with MP /HBOC vs. control.

4.5: Define principal drivers of inflammation following transplantation.

This was performed essentially as described for data on inflammatory mediators assayed in graft tissue during the period of preservation with MP /HBOC. We expected to find different inflammatory drivers in samples from transplanted tissues after having been preserved with MP /HBOC vs. control.

4.6: Define dynamic networks of inflammation following transplantation.

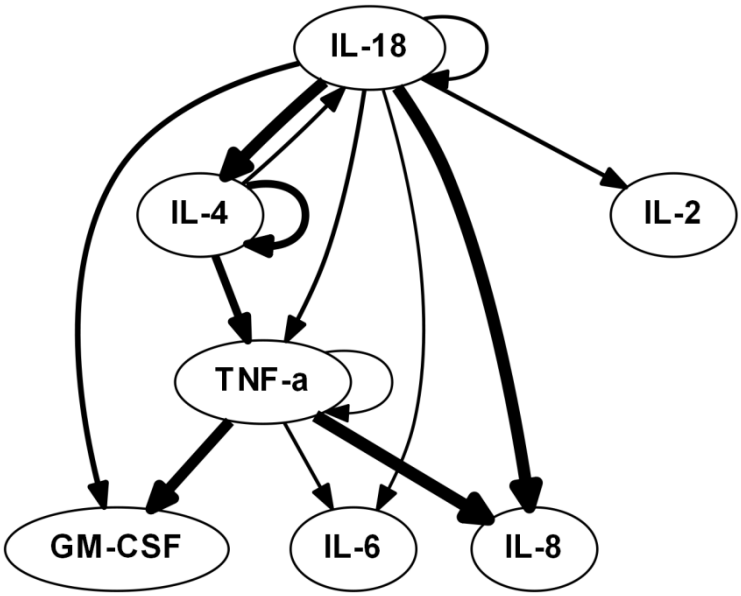
This was performed essentially as described for data on inflammatory mediators assayed in graft tissue during the period of preservation with MP /HBOC. We expected to find different networks of inflammatory mediators in samples from transplanted tissues after having been preserved with MP /HBOC vs. control.

4.7: Inflammatory markers

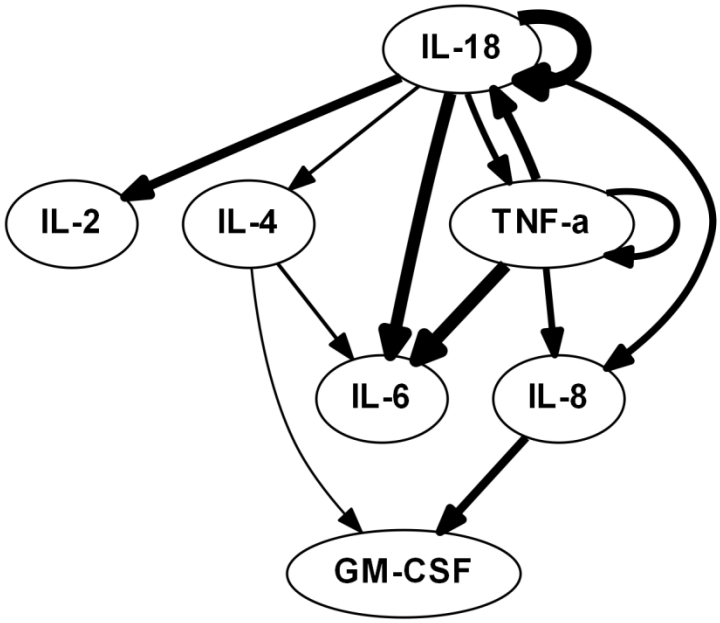
In order to quantify the inflammatory process triggered by IRI and the imminent alloreaction experienced by the VRAM after graft implantation, a full cytokine profile was obtained during VRAM graft preservation in both groups (MP and CSP). Subsequent samples were obtained from tissue biopsies during the post-operative period. Tissue and perfusate assays of interferon IFN-γ, IL-10, IL-12/IL-23 p40, IL-1β, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α were carried out using a Luminex™ beadset from Affymetrix (Santa Clara, CA). GM-CSF, IL- 1α, IL-1RA, IL-2 and IL-18 were measured using a Luminex™ beadset from Millipore (Merck KGaA, Darmsdadt, Germany)[13]. Tissue samples were normalized by protein content in order to account for experimental variability in cell number and protein concentration among individual samples. Standard statistical analysis (ANOVA) of the cytokines' concentration across time points did not show any significant difference between the two groups during the ex-vivo stage. This subsequent integrated system analysis utilizing PCA, DBN inference and DyNA provided by this DOD extension provided us with the opportunity to demonstrate the different inflammatory pathways experienced by the two groups regarding the preservation method over a 14 hour period.

We're able to demonstrate again, analogue to our previous liver experiments, a central role for TNF-α in the CSP group as major promoter for sustained and enhanced inflammation (Figure 1) as demonstrated by the initial DBN inference.

Control

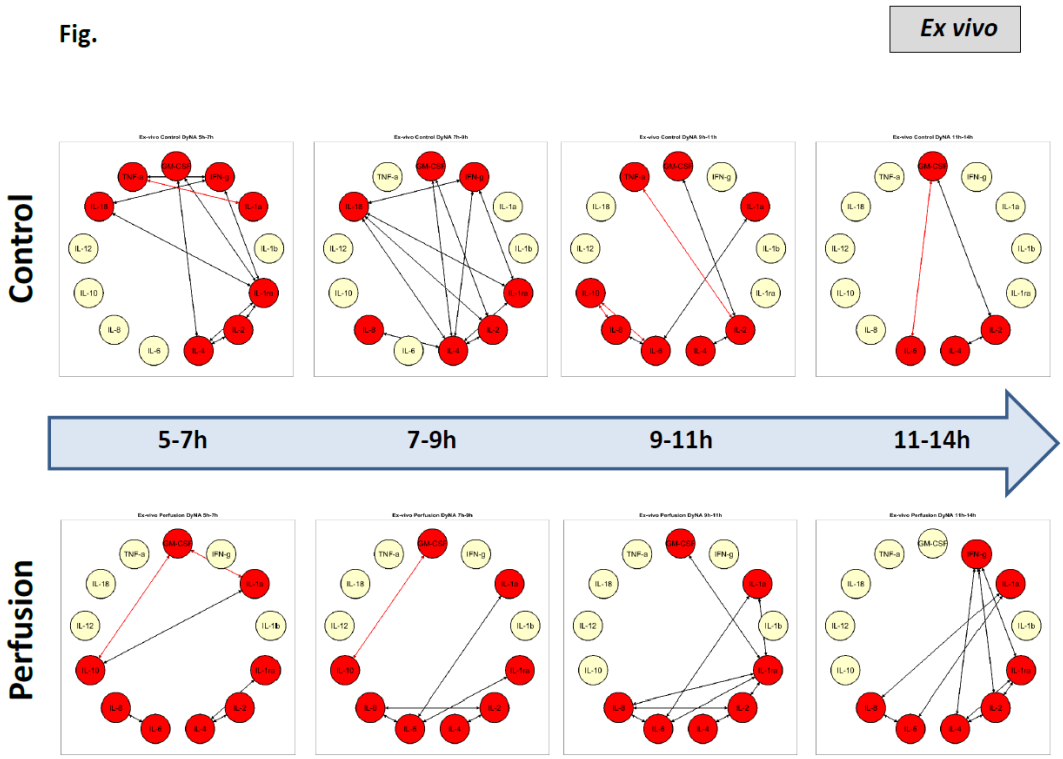


Perfusion



Subsequent dynamic network analysis (DyNA) showed a lower degree of complexity in the CSP inflammatory network in spite sustaining a similar amount of connections (Figure 2). Figure 5 displays a horizontal analysis of all interactions observed among all the measured cytokines.

Fig.



This analysis was extended to all horizontal interactions of the inflammatory markers in each time point. This analysis was also capable to reveal all positive (upregulation) and negative (downregulation) interactions among these inflammatory markers in a dynamic and rather precise manner. Furthermore, both groups (control and study) were able to be compared side-by-side in each time point and across the entire preservation period regarding the persistent interactions among all the inflammatory markers. This dynamic approach wasn't possible when standard statistical analysis was performed.

Subsequent DyNA revealed rather interesting inflammatory pathways overtime in both groups. This modality showed a leading role in TNF-α activation seen earlier in the control group, where subsequent INF-ɣ activation was also detected. This aggressive inflammatory pathway was very different than the one seen contemporaneously in the MP group, where a much simpler cytokine network was primarily driven by IL-4 and IL-6. Interesting enough, the histology analysis showed the early presence of contraction bands (CB) in the sarcomeres of the control group at the 4 hour mark, which remained anoxic and hypothermic for the entire duration of the experiment (14 hours). A similar histological pattern (CB) has been similarly described in the sarcomeres of the myocardium of patients with coronary artery disease in the period that precedes a well-defined ischemic myocardium infarct. Further analysis reveals the absence of any TNF-α in the MP group, showing the beneficial impact of effective ex-vivo oxygenation in this group (Figure 6). Previous clinical studies in patients with myocardium infarct (muscular damage from ischemia-reperfusion) have shown a major role in TNF-α and IL-1RA in the

initial upregulation of the inflammatory process following myocardium damage from prolonged ischemia [14].(Blancke F et al. Mediators of Inflammation, 2005; 6:385-89).

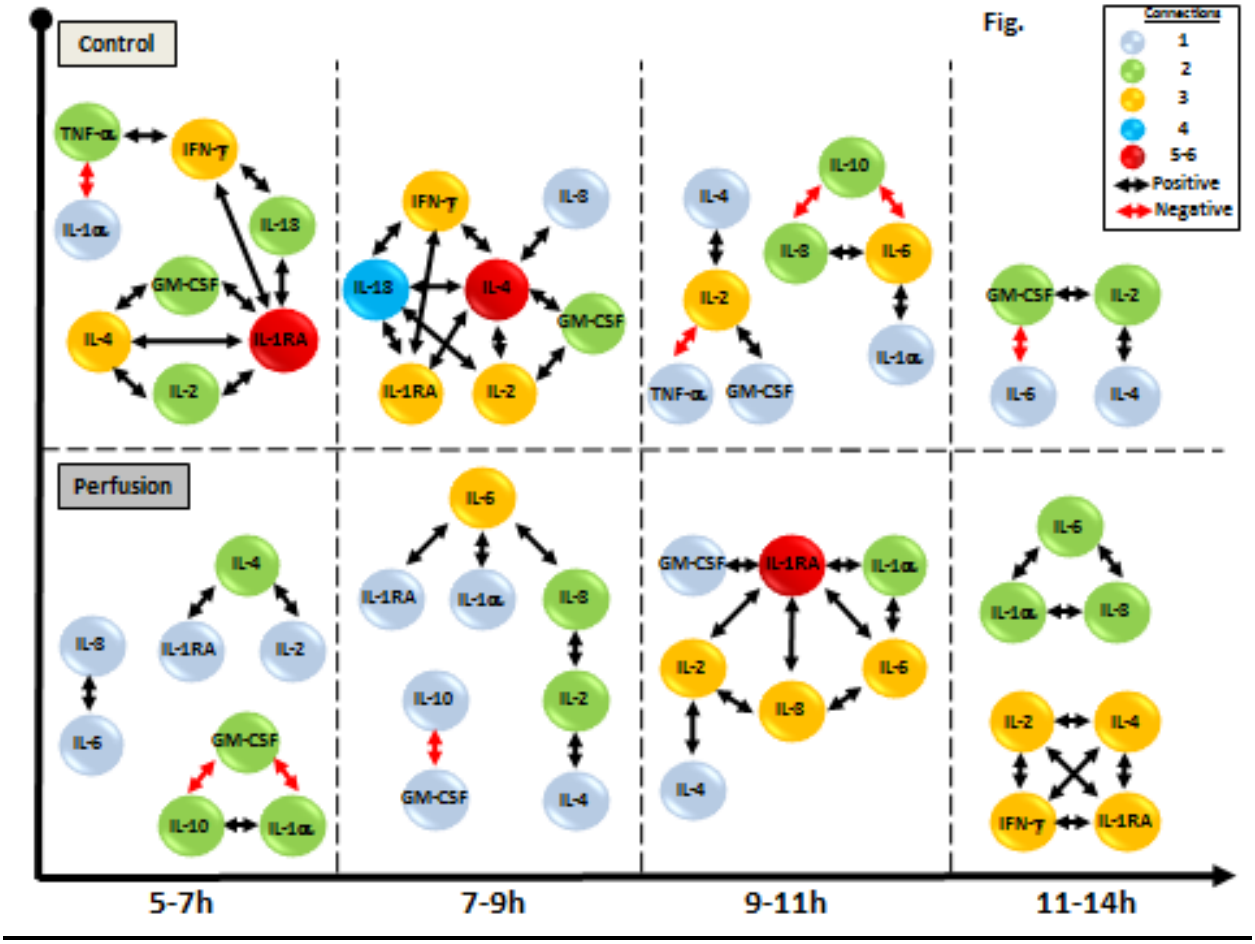


Figure 7 shows a comparative assessment overtime of the complexity of the inflammatory networks. In spite moving towards a lower number of connections, the control group (CSP) yielded a more robust ischemia-reperfusion injury after the VRAM implantation into the recipients. This is a very important finding that reinforces the need for extended assessment of IRIs after graft reperfusion, rather than a brief ex-vivo assessment by blood flush as previously seen in additional studies [15].

Fig.

Ex vivo

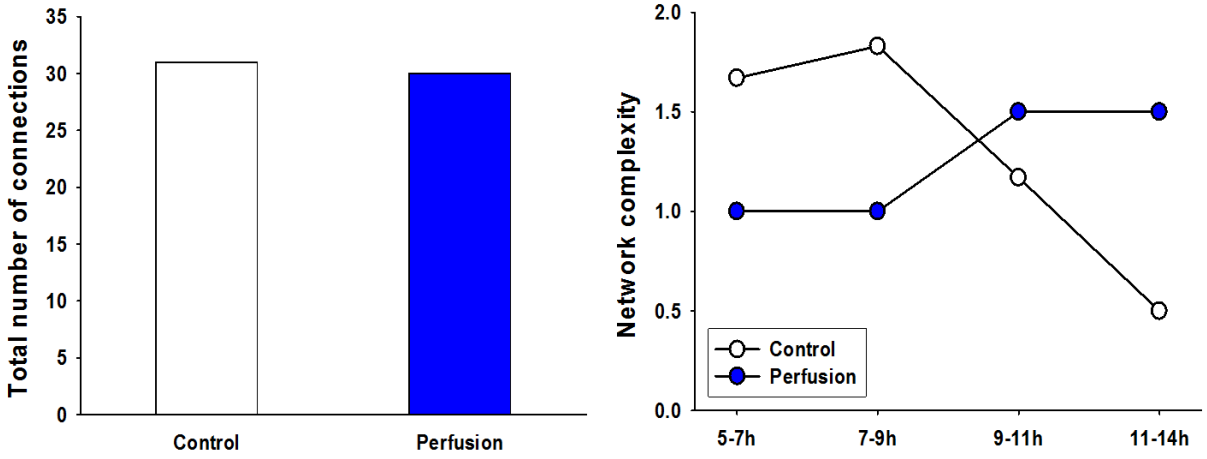
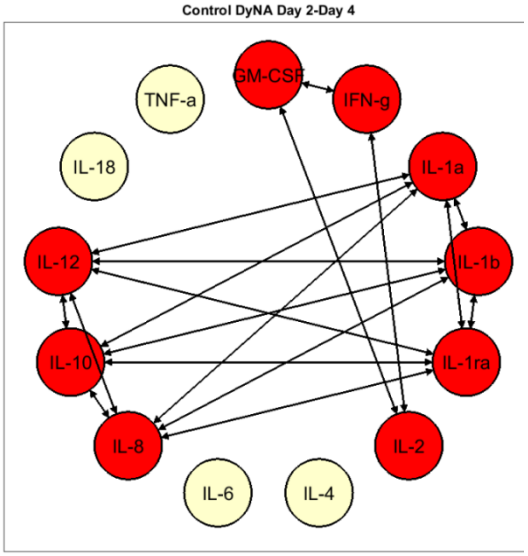
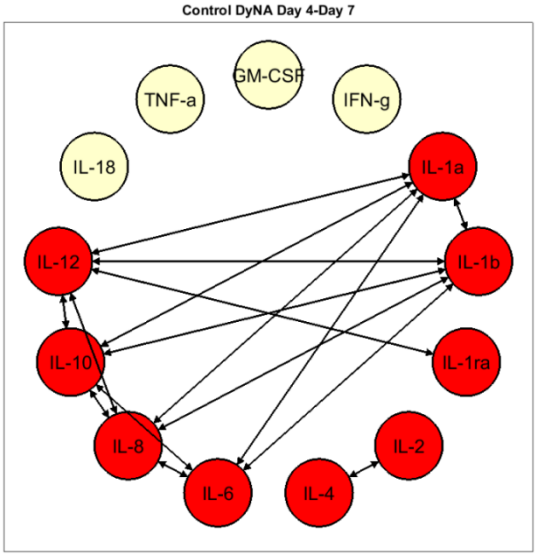
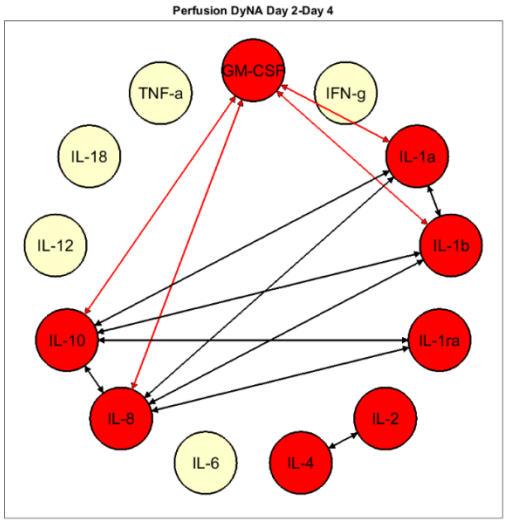
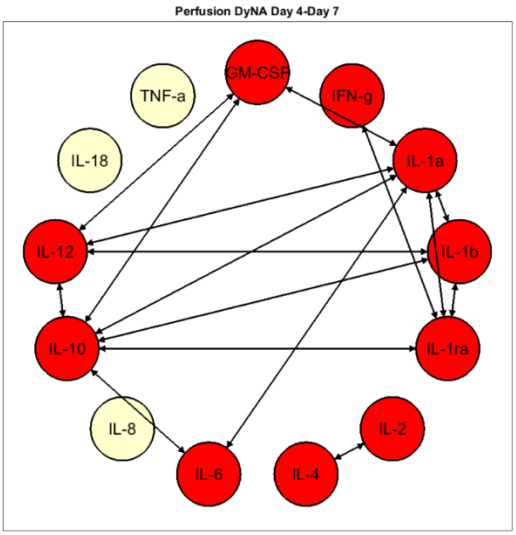


Figure 8 displays the evolution of the inflammatory network in-vivo after the VRAM implantation in the recipients, where both groups received similar triple immunosuppressive therapy (Prednisone, Tacrolimus and Mycophenolate) over a 7 day period. Similarly to the ex-vivo phase, the in-vivo data didn't show any significant difference between the CSP and the MP groups when standard statistical analyses (two-way ANOVA) were applied. When al data the data was combined and plotted in a dynamic fashion using DyNA tools, the control group showed an early upregulation of GM-CSF, which was followed by activation of both INF-ɣ and IL-2. These cytokines have been previously shown as potent mediators of early inflammation in patients undergoing controlled limb ischemia during elective procedures (Kamat P et al. Journal of Inflammation 2012; 9:18). Further immuneactivation was provided by upregulation of IL-1a, IL-1b and IL-1RA, stemmimg by direct upregulation from IL-12, IL-10 and IL-8. Interestingly, the MP group showed a different inflammatory pathway in-vivo during the first 4 days (2-4), where GM-CSF appeared to downregulate the activation of the same cytokines (IL-10, IL-8 and IL-1b). Histologically, the amount of inflammation experienced by the MP group was significantly lower after VRAM implantation into the recipients. As we extended our follow up towards post-operative days 4 to 7, the MP group showed a sustained GM-CSF interaction with IL-12, IL-10 and IL-1a (intra VRAM cytokine levels). Histologically, the MP group showed decreased inflammation, absence of acute cellular rejection and progressive recovery from mild IRIs. On the contrary, the CSP group showed an intra VRAM graft inflammatory pathway characterized by the absence of GM-CSF and INF-ɣ interactions, in a cytokine network with fewer overall connections when compared to the MP network. The CSP intra-graft profile appeared to sustain a close loop interaction between IL-1a and IL-1b, which leaked into additional activation of IL-12, Il-10, IL-8 and IL-6. Histologically, this inflammatory network was characterized by progressive muscle necrosis and a higher degree of early leukocyte infiltration. There

were clear signs of subsequent myofiber necrosis, myopathic changes in addition to edema and/or hemorrhage in the CSP group. Subsequent neutrophil infiltration and expanded areasof hemorrhage were clearly seen in the CSP group. The loss of muscle fibers due to hypereosinophilic degeneration was significant. Progressive granulomatous inflammation evolved overtime among a number of aggregated, large, activated macrophages and epithelioid macrophages. There were extensive necrotic areas with moderate to severe amount of inflammation on day 7 in the CSP group. Progressive neutrophilic infiltration was seen around extensive necrosis and loss of muscle fibers. Late macrophage infiltration (day 7) was also observed, leading into granulomatous-like changes resembling pseudo-abscesses surrounded by epithelioid macrophages, or multinucleated giant cells. This extensive muscle fiber degeneration evolved into further mineralization within aggregates of multinucleated giant cells.

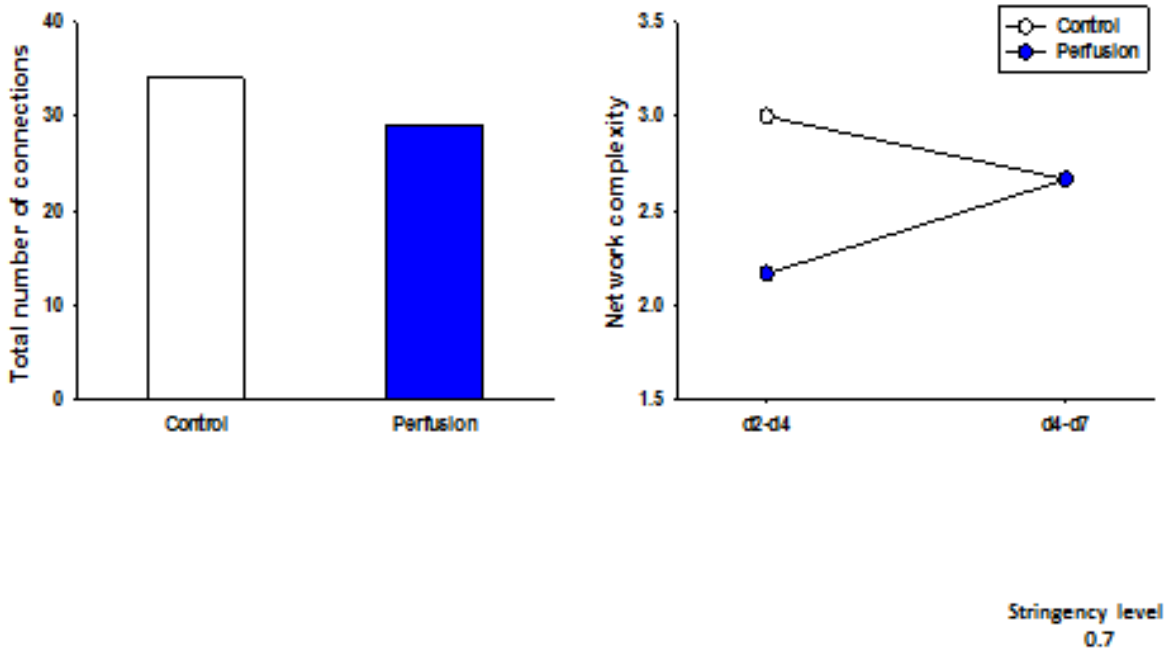
In-vivo assessment of cytokines (VRAM grafts) – Stringency level 0.7		
Group	**Days 2-4**	**Days 4-7**
Control (CSP)		

Study (MP)		

Similarly to the data from the ex-vivo stage, the complexity of the cytokine network appeared to have a bimodal behavior, where the level of complexity increased in the MP group overtime while the level of complexity decreased in the CSP over the 7 day period.

Fig.

In vivo



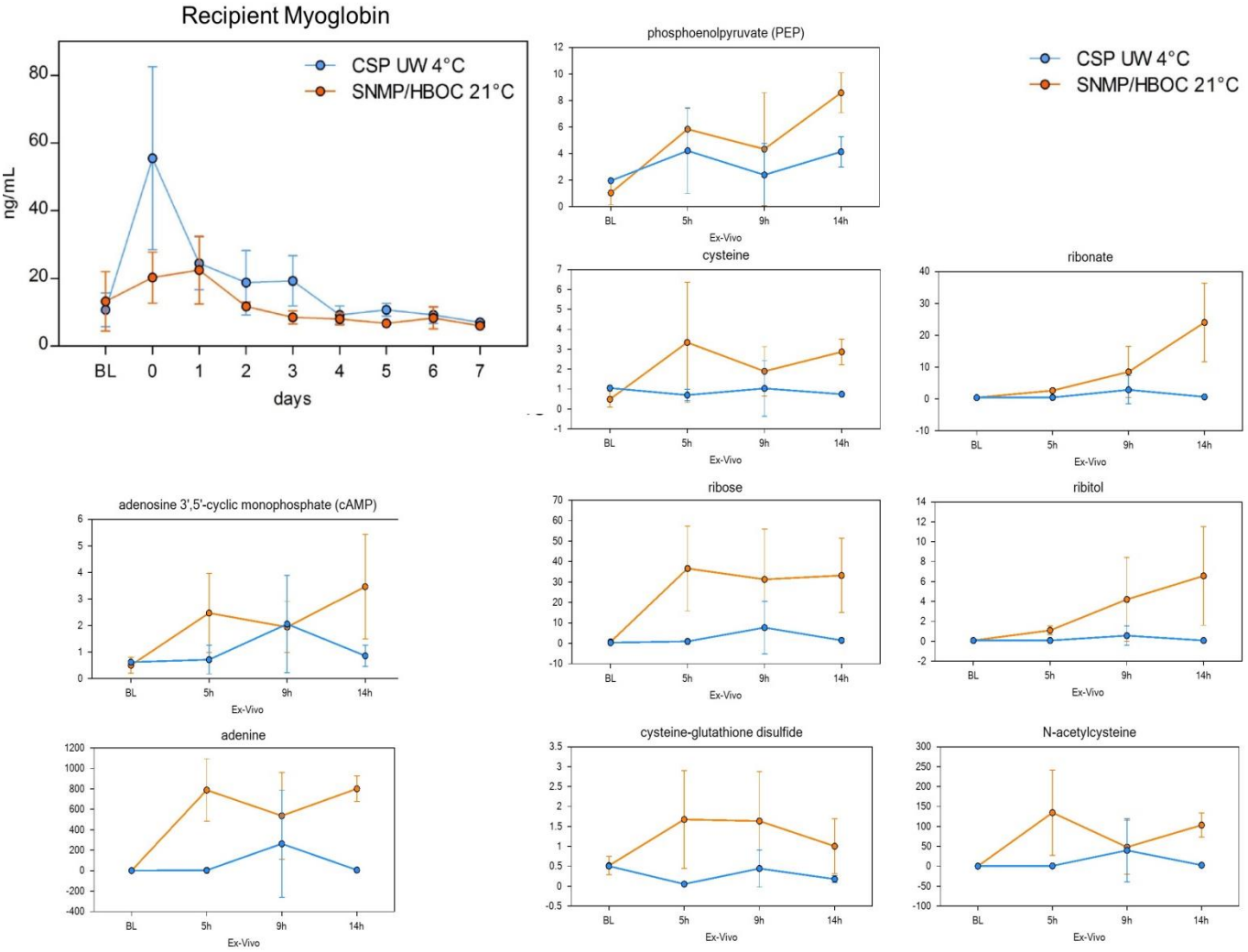
Conclusions:
This extended preservation period in a pre-clinical large animal model (swine) appeared to induce significant oxidative cell damage in the CSP group. Well established cytokine pathways (e.g. TNF-α and IL-1 upregulation) were initially seen in the CSP group. Histologically, significant neutrophil followed by macrophage infiltration were extensively documented in the CSP group, where significant muscle necrosis lead to irreversible muscle fiber degeneration followed by diffuse mineralization surrounded by aggregates of multinucleated giant cells (pseudo-abscess like lesions). This subsequent dynamic analysis of the inflammatory markers using PCA, DBN and DyNA tools was able to establish a more reliable link to the significant differences between the 2 groups documented by the initial histological analysis.

Metabolomics
Tissue samples from the VRAM grafts were obtained during preservation (0, 5, 9 and 14 hours) and after the transplant procedure on POD 0, 2, 4 and 7. These tissues were immediately frozen (OCT) and further submitted to Metabolon Inc., Raleigh, NC for metabolomics analysis. Following receipt, samples were inventoried, and immediately stored at -80^{o}C. At the time of analysis samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS platforms. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples. There were 653 compounds analyzed. Following log

transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch's two-sample *t*-tests were used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (*q*-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. The *q*-value describes the false discovery rate; a low *q*-value ($q<0.10$) is an indication of high confidence in a result. While a higher *q*-value indicates diminished confidence, it does not necessarily rule out the significance of a result.

Results: The VRAM grafts in the MP system sustained an intact energetic metabolism fueled by glucose over the 14 hour period of preservation when compared to the CSP group. MP sustained normal skeletal muscle glycolysis as evident by higher: glucose 6-phosphate (↑23 fold, p=0.01), fructose-6-phosphate (↑12 fold, p=0.03), and phosphoenolpyruvate (↑8 fold, p=0.03). Nucleic acid synthesis was significantly higher in the MP flaps: cysteine (↑3.88 fold, p=0.02), ribose (↑22 fold, p<0.001), ribonate (↑37 fold, p<0.001), and ribitol (↑10.5 fold, p<0.001). MP led to significantly higher levels of reactive oxygen species (ROS) scavengers in the VCAs: glutathione-cysteine disulfide (↑5.6 fold, p=0.01) and N-acetylcysteine (↑40 fold, p=0.007). Furthermore SNMP provided sufficient energy precursors and metabolites: adenine (↑129 fold, p=0.002), cAMP (↑3.7 fold, p=0.02), AMP (↑3.2 fold, p=0.01) and 3'-AMP (↑2.3 fold, p=0.01). CSP grafts faced extensive amino acid metabolism dysregulation as suggested by significantly higher levels of: N^6-succinyladenosine (p<0.01), valine (p<0.01), 2-methylbutyrylcarnitine (p=0.01), 3-hydroxyisobutyrate (p=0.01), and ethylmalonate (p=0.009) tissue levels. Glycogen reserves were higher in the MP group. There was adequate glucose supply in the MP and no signs on glycogen breakdown. The pentose metabolites were significantly higher in the MP group, showing a higher anabolic state when compared to the CSP group. The CSP group appeared to have a sustained catabolic state when compared to the MP group. There were signs of higher production of nucleotides and nucleic acids precursors in the MP. As previously seen in our experience with liver allografts under the MP/HBOC system, there was a significant (30 fold higher) increase in the metabolic pathways related to cell regeneration once oxygenation was effectively provided ex-vivo during preservation. There were signs of higher production of aromatic amino acids in the MP group when compared to the CSP group. The MP/HBOC system provided more effective anti-oxidant pathways when compared to the CSP group. There were higher levels of end-products from oxidized stress in the MP group, which can be seen as an indirect sign of lower stress from less significant IRIs when compared to the CSP group. Contrary to our previous experience with livers, the VRAM grafts under the MP/HBOC system showed lower fatty acid β-oxidation when compared to the CSP group. This means a lower of fatty acids into the mitochondria as a source of fuel. This also favors our initial findings regarding the preferential pathway for glucose as the primary source of energy in striated muscles. Further analysis of the purine metabolism (adenine components) showed indirect signs of higher ATP production in the MP group when compared to the CSP. The adenine family has a variety of roles in cellular respiration and protein synthesis. There were higher levels of cAMP in the MP group, showing higher ATP production in this group. AMP is used as a monomer in RNA synthesis. The cAMP as a derivative of ATP has a significant role in signal transduction. SNMP increased fatty

acids (FAs) Ω-oxidation pathway as evident by significantly higher tissue levels of dicarboxylic FAs: 2-hydroxyglutarate (↑4.8 folds, p=0.02), adipate (↑5.55 folds, p=0.003), and 2-hydroxyadipate (↑2.5 folds, p=0.01). Reduction of β-oxidation was evident by substantial increase in the levels of acyl-carnitine metabolites in the SNMP/HBOC grafts: cis-4-decenoyl carnitine (↑8 folds, p=0.04), laurylcarnitine (↑8.3 folds, p=0.02), oleoylcarnitine (↑9.5 folds, p=0.01), myristoleoylcarnitine (↑22 folds, p=0.006), and adipoylcarnitine (↑7 folds, p<0.001). This was mirrored by significantly higher levels of end-products of β-oxidation pathway in the CSP group as shown by 4-hydroxybutyrate (p<0.01). Cellular membrane integrity was well preserved histologically and further evidenced by significantly higher levels of phospholipids in the SNMP/HBOC group: oleoylcholine (↑5 folds, p<0.01) and choline (↑3 folds, p=0.01). In addition, early signs of myopathy were observed with significantly higher tissue levels of butyryl-carnitine (p=0.04) in the CSP grafts, which were further corroborated by histopathologic analysis.



Conclusions: The MP/HBOC system can effectively preserve VRAM allografts when compared to CSP. MP significantly mitigates IRI, which was manifested earlier within the first 4 hours in the CSP group. Effective *ex-vivo* oxygenation with HBOC decreases post-transplant inflammation in skeletal muscle fibers and upregulates regenerative metabolic pathways driving early recovery from IRI. There is a similar up-regulation of TNF-α in the CSP group, which is similar to our previous data obtained in liver allografts after a period of 9 hours of preservation. Effective ex-vivo oxygenation with the MP/HBOC system avoids the early (hours) formation of hypercontracted sarcomeres (CB) and the subsequent development (days) of myofiber necrosis, myopathic changes, edema and hemorrhage seen extensively in CSP as the current standard of care. The significant IRI observed in the CSP group yielded a significant hypereosinophilic sarcomere degeneration leading into irreversible loss of muscle fibers, followed by progressive granulomatous inflammation accompanied by the infiltration of large, activated macrophages, epithelioid macrophages and multinucleated giant cells, leading into terminal mineralization and complete loss of muscle mass. Metabolic precursors of nucleotide synthesis were significantly upregulated in the MP/HBOC group. These precursors appear to be implicated in a strong regenerative response elicited by effective oxygenation of skeletal muscle, which also has a positive impact in energy utilization and ROS scavengers. The MP/HBOC also promoted effective *ex vivo* oxygenation and shifted skeletal muscle metabolic profile from β-oxidation towards Ω-oxidation during VCA preservation when compared to the prolonged anoxia under hypothermic conditions induced by CSP. This can be interpreted as a sign of mitochondrial dysfunction experienced by the CSP group. In fact, Ω-oxidation is linked to balanced redox state and less oxidative damage during stressful conditions induced by these experiments. In contrast, CSP appears to increase a reactive skeletal muscle β-oxidation pathway, which leads into oxidative damage and disintegration of cellular membranes when prolonged hypothermia, anoxia, and limited glucose supply is imposed. Contrary to the CSP group, MP/HBOC protects skeletal muscle against early graft myopathy. These complex metabolic features seen in the both the muscle and adipose tissue were extensively corroborated by the serial histological findings, revealing in a close analogy the same protective role exerted by effective ex-vivo oxygenation documented extensively in our previous liver experiments.

5. Products/Publications/Presentations

A new device for limb perfusion was developed through these experiments. We're able to implement new changes to our original prototype that rendered the filing on a new patent for the new Limb Assist® device to be produced by Organ Assist, Groningen, Netherlands.

The new CTA MP device developed at the MIRM/U Pitt has the following features:

1. The new PVC medical mesh placed horizontally in the limb perfusion chamber (LPC).
2. The VRAM graft placed horizontally and in an anatomic position within the LPC.
3. Free gravity drainage through the PVC mesh from the VRAM graft

4. Pulsatile flow in the infusion port. The changes in the software are specified in a separate section.

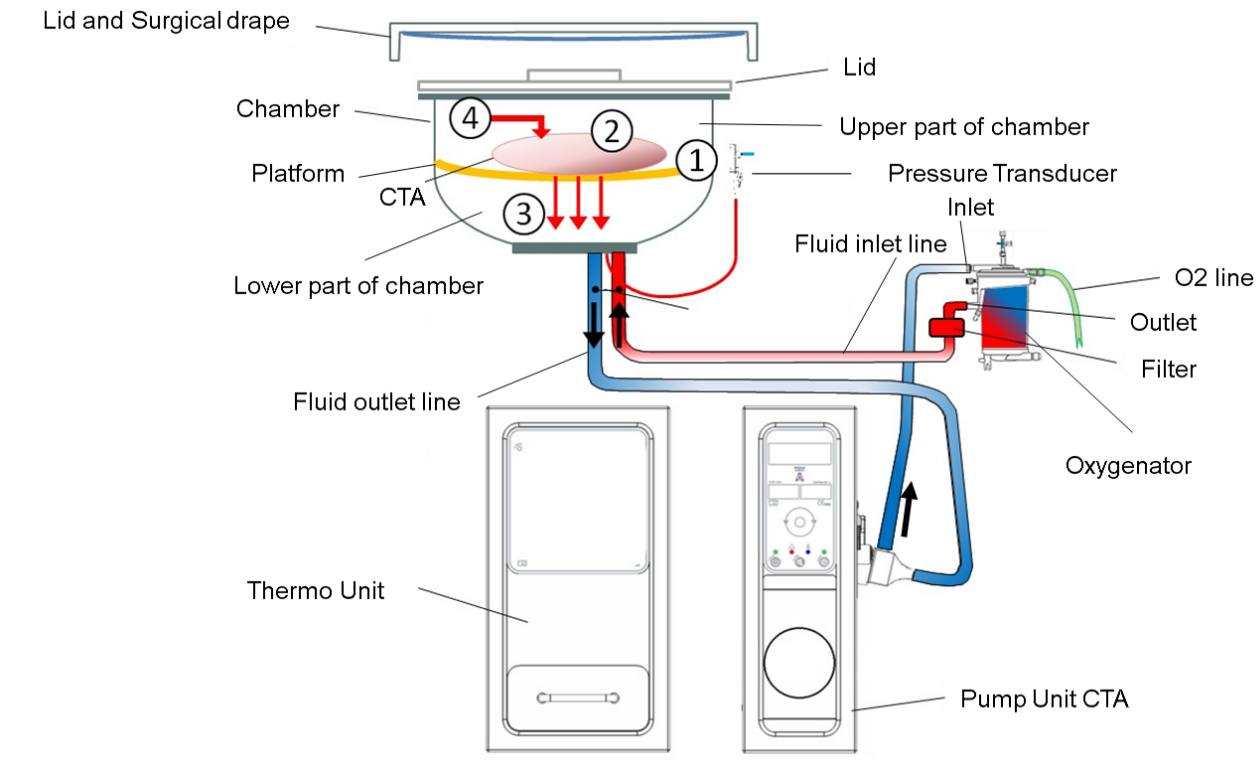


Figure 22

5.1. Publications

1. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, Fontes PA. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. 17th Congress of the European Society for Organ Transplantation, Brussels, Belgium, September 2015.
2. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, Fontes PA. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. Jahreskongress Schweizerische Gesellschaft für Plastische, Rekonstruktive und Ästhetische Chirurgie (SGPRAC), Thun, September 11-12 2015.
3. Schweizer R, Oksuz S, Komatsu C, Gorantla VJ, Fontes PA. Subnormothermic Machine Perfusion with Hemoglobin-based Oxygen Carriers for Tissue Preservation in Vascularized Composite Allotransplantation. Jahreskongress Deutsche Gesellschaft für Plastische, Rekonstruktive und Ästhetische Chirurgie (DGPRAC), Berlin, October 1-3 2015.

4. Oksuz S, Schweizer R, Minervini M, Banan B, Gorantla V, Fontes P. Subnormothermic Machine Perfusion (SNMP) with a Novel Hemoglobin-based Oxygen Carrier (HBOC) Solution for ex vivo Preservation in Vascularized

Composite Allotransplantation (VCA). American Transplant Congress Boston, MA, June 2016

5. Banan B, Schweizer R, Oksuz S, Plock J, Gorantla V, Fontes P. Sub-normothermic machine perfusion (SNMP) of vascularized composite allografts (VCA) with a novel hemoglobin oxygen carrier (HBOC) solution promotes effective ex-vivo oxygenation, mitigates ischemia reperfusion injury and upregulates regenerative responses in skeletal muscle. Military Health System Research Symposium, Kissimmee, FL August 2016.
6. Banan B, Schweizer R, Oksuz S, Plock J, Gorantla V, Fontes P. Support of Cell Membrane Integrity, Reduction of Early Myopathy and Decrease in Fatty Acid β-oxidation in Skeletal Muscles of Vascularized Composite Allografts (VCA) during Subnormothermic Machine Perfusion (SNMP) using a Novel Hemoglobin Oxygen Carrier (HBOC) Solution. Military Health System Research Symposium, Kissimmee, FL August 2016.
7. Fontes P, Gorantla V, Plock J, Davis M. Ex-vivo perfusion of vascularized composite allografts at 21°C with a hemoglobin-based oxygen carrier (HBOC) solution over 14 hours: new opportunities for effective en-route care. NATO Symposium on "Regenerative Medicine and advanced Rehabilitation - Today and in Future" (HFM-272) October, 2016, Brussels, Belgium.

5.2. Presentations

1. Invited speaker - 61st Meeting of the American Society of Artificial Organs. Perfusing the Kidneys and Tissue Allografts Outside of the Body, Chicago, IL, July 2015

2. Invited speaker – Summer School, McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA. The new ex-vivo world; perfusing human organs outside of the body. July 2015

3. Invited speaker – Regenerative Surgery – The Cutting Edge. Organ Preservation: The New Frontier in Organ transplantation. LifeNet Health, Virginia Beach, VA. September 2015.

4. Invited speaker – 1st Annual Meeting of the Society for the Advancement of Transplant Anesthesia, University of Pittsburgh. Organ Preservation Methods. Pittsburgh, PA, September 2015.

5. Invited speaker – Continuing Education Symposium, CORE, Pittsburgh, PA – Organ Preservation: The New Frontier in Organ transplantation. CORE, Pittsburgh, PA, October 2015.

6. Invited speaker – Henry Ford Hospital – Surgical Grand Rounds. The new ex-vivo world: extracorporeal perfusion of organs and composite tissues. Detroit, MI. February 2016.

7. Invited speaker – The Robert H. Ivy Society for Plastic Surgeons – 62nd Scientific Meeting. The new ex-vivo world: extracorporeal perfusion of organs and vascularized composite allografts. Pittsburgh, Pittsburgh, PA. April 2016.

8. Invited speaker – Annual Critical Care Nursing Symposium. The new ex-vivo world: extracorporeal perfusion of organs and composite tissues. Pittsburgh, Pittsburgh, PA. April 2016.

9. Invited speaker – OptumHealth Education, University of Pittsburgh Medical Center (UPMC) Transplant Services and Children's Hospital of Pittsburgh of UPMC. "History of Transplant Excellence, A Future of Innovation".The new ex-vivo world: extracorporeal perfusion of organs and composite tissues. Pittsburgh, Pittsburgh, PA. May 2016.

10. Oral presentation – TechConnect World Innovation, National Innovation Summit and National SBIR/STTR Conference. Human-derived hemoglobin oxygen carrier. Gaylord National Resort and Convention Center, National Harbor, Maryland, USA. May 2016.

11. Invited speaker – Summer School, McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA. The new ex-vivo world; perfusing human organs outside of the body. July 2016

12. Invited Speaker – XLIII Annual Congress of the European Society for Artificial Organs. The new ex-vivo world: machine perfusion with a new hemoglobin based oxygen carrier solution. Nalecz Institute of Biocybernetics and Biomedical Engineering (IBBE PAS) and the International Centre of Biocybernetics, Polish Academy of Sciences, Warsaw, Poland. September 2016.

6. Inventions/Patents/Licenses

Pitt Ref. No. 03677
Klarquist Ref. No. 8123-95565-01
For **DEVICE FOR COMPOSITE TISSUE ALLOTRANSPLANT PRESERVATION AND USE THEREOF**
Filed September 11, 2015
Provisional Patent Application No. 62217565
EFS ID: 23468151
Confirmation number: 9459
Country: U.S.

7. Reportable Outcomes

This extension has allowed us to extend our initial analysis of the inflammatory markers involved in the ischemia-reperfusion processes induced by this pre-clinical large animal model involving extended preservation of vascularized composed allotransplants (VCA). In spite finding significant differences in clinical and histological analysis, the initial assessment of the inflammatory markers provided by standard statistical analysis (two-way ANOVA) was inconclusive and unrelated to the additional findings.

Our preliminary experience with this sophisticated analytical approach has suggested that analysis of dynamic inflammation networks in the setting of liver preservation may identify novel diagnostic and therapeutic modalities.

This similar analytical approach (e.g. PCA, DyBN and DyNA) utilized in this extension has given us new insights about the dynamic interaction of the inflammatory markers involved in this CVA model where porcine VRAM grafts were preserved for 14 hours before transplantation. Additional intra-graft studies (obtained from tissue biopsies) revealed additional mechanistic nuances in the complexity and dynamic horizontal interactions among the inflammatory markers initially assessed in our original study. The information obtained with this extension wil enhance the quality of the final manuscript that will be published after the completion of this DOD-sponsored project, where a new medical device has been conceived and where new analytical tools have been applied as a way to enhance the knowledge of the biological variables involved in ischemia reperfusion injuries on VCAs.

7. Reportable Outcomes

These proof-of-concept pre-clinical experiments in a porcine model were very important to determine the role of MP as a new modality for CTA preservation. We’re able to reproduce the successful findings published from our liver experience in a similar MP/HBOC system utilized over a 9 hour period.

The MP/HBOC system as a new option for CTA preservation displayed the same safety and effectiveness seen in the solid organ model.

The reported experiments were based on a very challenging model involving 14 hours of CTA preservation followed by graft implantation and the subsequent follow up of the transplant recipients under triple immunosuppressive therapy. The control group VRAM grafts were preserved within the current standard of care (CSP) and showed moderate to severe IRIs with massive necrosis within the first post-operative week.

The MP-treated VRAM grafts had a significantly better outcome, based on the lower magnitude of the IRIs, lower degree of inflammation and additional signs of advantageous metabolic features regarding oxidative stress, fuel utilization and protein synthesis.

The new Limb Assist® device will be utilized on a follow up DOD project (MR140030) led by LtCol Michael Davis, MD, FACS, USAF, MC in the Institute for Surgical Research, San Antonio, TX.

Our specific aims were fully achieved. In summary:

1. The MP/HOBC system promoted an increased CIT period without any damage to the VRAM graft during preservation
2. The MP/HBOC system minimized the IRIs observed during preservation when compared to the current standard of care (CSP).
3. The MP/HBOC system had a positive impact on the immune profile of the VRAM grafts, where a significantly lower degree of inflammation was observed after preservation. This should have a major impact in long term studies where the lesions acquired from IRIs would have a lower degree of alloactivation and most likely a lower degree of both acute and chronic rejection.

8. Other Achievements

The interactions experienced during the data analysis of these experiments have enhanced our links to the ISR, San Antonio, TX and the Department of Plastic Surgery, Zurich, Switzerland. Additional interactions with the Center for Inflammation and Regenerative Modeling (CIRM), University of Pittsburgh, have yielded a great collaborative effort towards the use of computational biology tools for the inflammatory process seen earlier after graft preservation. We intend to proceed with additional studies with the current cytokine data and a further request for this activity will be sent after this report.

We faced a problem with sample storage that further implicated in the quality of the micro array data to be generated from this study. The micro array studies have been dropped.

9. References

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10. APPENDICES:

QUAD CHARTS:

Response to Fund Opportunity No. W81XWH-13-2-0061
Technology Area of Interest: Transplantation
Proposal Title: Ex-vivo machine perfusion in CTA with a novel oxygen carrier system to enhance graft preservation and immunologic outcomes

PI: Paulo Fontes, MD, FACS **Org:** University of Pittsburgh **Award Amount:** $40,000 (extension)

Objective(s)
Our objective in this extension was to conduct a comprehensive analytical study with computational biology tools to define the principal drivers of inflammation as well as interconnected inflammatory networks. This study was originally designed to improve the functionality of the CTA tissues after transplantation. The central hypothesis was to demonstrate the benefits of machine perfusion and ex-vivo oxygenation during the graft preservation period.

Study/Product Aim(s)
1; to define principal drivers and networks of protein-level inflammatory mediators as related to the effect of MP/HBOC on grafts during the preservation period.; 2; to define principal drivers and networks of protein-level inflammatory mediators as related to the effect of MP/HBOC on the immune profile of various flap tissues after transplantation.

Approach:
To define the principal drivers of inflammation during CTA preservation with MP/HBOC and after transplantation we carried out Principal Component Analysis of all data. Dynamic Bayesian Network (DyBN) inference and Dynamic Network Analysis (DyNA) were subsequently used to define networks of cytokines and chemokines models for the evolution of the probabilistic dependencies within a system over time.

This extended preservation period in a pre-clinical large animal model (swine) appeared to induce significant oxidative cell damage in the CSP group. Well established cytokine pathways (e.g. TNF-α and IL-1 upregulation) were initially seen in the CSP group. This subsequent dynamic analysis of the inflammatory markers using PCA, DBN and DyNA tools was able to establish a more reliable link to the significant differences between the 2 groups documented by the initial histological analysis.

Timeline and Cost

Activities	2015-16	1Q	2Q	3Q	4Q
Infrastructure- Development of research protocol and budget. Organization of personnel and resources.					
Research- Rerunning all the previous data through PCA, DBN and DyNA protocols.					
Data Management– Analyze all cytokines' horizontal and vertical interactions.					
Analysis and Reporting– Determine the impact of significance of the dynamic interactions among all inflammatory markers.					
Estimated Budget ($500K)		$000	$	$	$40k

Goals/Milestones

1Q Goals
- Development of research protocol and budget for extension study.

2Q Goals
- Applying PCA, DBN and DyNA analytical tools to previous data
- Define Stringency level (0.7) range for all interactions
- Develop graphic representations for ex-vivo and in-vivo data

3Q Goals
- Complete all data analysis and compare results with previous histology data

4Q Goals
- Complete final report

Comments/Challenges/Issues/Concerns –
- The overall findings were similar to previous liver experiments regarding the leading role of TNF-α in ischemia-reperfusion injuries.

Budget Expenditure to Date
Projected Expenditure:$40,000; Actual Expenditure: $35,000